KINETIC STUDIES OF ALLOSTERIC PHOSPHOTRANSFERASES

A THESIS

submitted for the Degree

of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

VERN LEE SCHRAMM

October, 1968

This thesis embodies the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from October, 1965 to October, 1968, during the tenure of an Australian National University Research Scholarship.

STATEMENT

The regulations of the Australian National University require that a statement be made describing which parts of the work in this thesis have been carried out by myself.

All experimental results described in this thesis were obtained by myself, while the derivation of rate equations was done in collaboration with Dr. J.F. Morrison of this department.

Candidate's Signature:

Vern Schramm

ACKNOWLEDGEMENTS

It is a pleasure to thank my supervisor, Dr. J.F. Morrison, for his guidance and encouragement during this work, and for his interest and readiness for discussion at any time.

I wish to thank both Dr. R.L. Blakley and Professor Frank Gibson for the privilege of working in the department; and to express my appreciation to the members of the department for the help they have given me.

I am grateful to Mrs. M. Labutis and Mr. Bruce E. Kemp for their skilful and patient technical assistance. I also wish to thank the Head Technicians, Mr. I. Reid and Mr. A. Howkins, for their co-operation in many matters.

The photographic prints for this thesis were prepared by Mr. R. Westen and staff, whose help is greatly appreciated, and I wish to thank Mrs. N. Aldrich for preparation of lettering for the illustrations. I am also thankful for the rapid and accurate typing of Mrs. C. Withers, which has been of great assistance in preparing this thesis. Further, I wish to thank Mrs. N. Young and staff of the University Typing Scheme for the final printing of this thesis.

I am also very grateful for the award of an Australian National University Scholarship during the tenure of which this work has been carried out.

PREFACE

Figures and tables in the Introduction are identified by Arabic numerals only, while those in the following Chapters (I-IV) consist of two parts : Roman numerals indicating the chapter, and Arabic numerals the number of the figure or table within the chapter.

Abbreviations used in this thesis are :

AMP, ADP, ATP	adenosine mono, di and triphosphate
CDP, CTP	cytidine di and triphosphate
GDP, GTP	guanosine di and triphosphate
IMP, IDP, ITP	inosine mono, di and triphosphate
UDP, UTP	uridine di and triphosphate
dTDP, dTTP	deoxythymidine di and triphosphate
dGTP	deoxyguanosine triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
TPP	thiamine pyrophosphate
NDP, NTP	nucleoside di and triphosphate
$MNDP^-$, $MNTP^{2-}$	metal complexes of nucleoside di and triphosphates
$MgdNTP^{2-}$	magnesium deoxynucleotide triphosphate
Pi	inorganic phosphate
EDTA	ethylenediaminetetraacetic acid
DEAE-	diethylaminoethyl-

CM-	carboxymethyl-
NAD, $NADH_2$	oxidized and reduced nicotinamide-adenine dinucleotide

TABLE OF CONTENTS

INTRODUCTION

General Introduction

The work reported in this thesis is concerned with kinetic studies of the reaction catalyzed by an allosteric enzyme, nucleoside diphosphatase, and was undertaken in an attempt to gain some insight into the mechanism of the reaction. By way of a background to the results obtained, the Introduction deals with the present state of knowledge in regard to allosteric enzymes.

In the General Introduction will be presented general and historical information pertaining to allosteric enzymes under the sections : (1) The discovery of allosteric enzymes. (2) Criteria for classifying enzymes as being allosteric. and (3) Function of allosteric enzymes.

The discovery of allosteric enzymes

In the late 1950's and early 1960's it became apparent that many enzymes which could be considered to hold key positions in metabolic pathways were inhibited or activated by some intermediate of or by the final product of that specific pathway. Further, it was noted that these compounds did not necessarily have a structural resemblance to the substrate(s) or product(s)

of that particular enzyme reaction (Helmreich and Cori, 1964). In addition to the activation and/or inhibition of these enzymes by reaction pathway intermediates, a further feature which was frequently observed was the sigmoidal or "S" shaped plot of initial velocity against substrate concentration. This sigmoidal plot was in marked contrast to the hyperbolic plot obtained for enzymes which conformed to the Michaelis-Menten (1913) formulation.

In 1961, Monod and Jacob referred to this type of enzyme as being "allosteric", and since that time many enzymes have been added to this group. As an indication of the rate of discovery of allosteric enzymes, it may be noted that only 12 had been recognized in 1961 (Umbarger, 1961); 24 were listed by Monod, Wyman and Changeux in 1965; while Table 1 lists the 50 allosteric enzymes which are known at the present time.

After enzymes exhibiting allosteric properties had been described, activators and inhibitors became known by various names such as allosteric effectors, allosteric modifiers, and allosteric ligands. However, in this paper, all allosteric reactants will be referred to as allosteric modifiers after the nomenclature of Frieden (1964).

TABLE 1. : Enzymes which exhibit allosteric properties[a].

Enzyme	Activator	Inhibitor	Kinetic Properties[c]	Subunit Structure[d]	Reference
Acetyl-CoA carboxylase	Citrate	?[b]	Non-Linear	+,>10	Vagelos et al. (1963) Gregolin et al. (1968)
N-acetylglutamate 5-phosphotrans-ferase	?	Arginine	Non-Linear	?	Farago & Denes (1967)
Adenine phospho-ribosyltrans-ferase	?	GMP	Non-Linear	?	Hori et al. (1967)
Adenylate deaminase	ADP,ATP	GDP,GTP	Non-Linear	?	Smiley & Suelter (1967)
ADP-D-Glucose pyrophosphorylase	Fructose-1,6-diphosphate glyceraldehyde 3-phosphate	?	Non-Linear	?	Preiss et al. (1965)
AMP nucleosidase	ATP	Pi	Non-Linear	?	Yoshino et al. (1967)
Aspartic trans-carbamylase	ATP	CTP	Non-Linear	+,3 or 4	Changeux et al. (1968) Weber (1968b)
Aspartokinase	?	Threonine	Non-Linear	?	Stadtman et al. (1961)
Aspartokinase	?	Lysine	Non-Linear	?	Stadtman et al. (1961)
Carbamyl phos-phate synthetase	Ornithine	CTP	Non-Linear	?	Pierard (1966) Kerson & Appel (1968)
CDP-D-Glucose pyrophosphorylase	?	CDP-paratose	Non-Linear	?	Mayer & Ginsburg (1965)

TABLE 1. : cont'd.

Enzyme	Activator	Inhibitor	Kinetic Properties[c]	Subunit Structure[d]	Reference
Chorismate mutase	?	Tyrosine	Non-Linear	?	Cotton & Gibson (1965, 1967)
dCMP deaminase	dCTP	dTTP	Non-Linear	+, ?	Maley & Maley (1965)
CTP synthetase	GTP,CTP	?	Non-Linear	?	Long & Pardee (1967)
3-Deoxy-D-arabino-heptulosonic acid synthetase	?	Tryptophan	Non-Linear	+, ?	Doy (1968)
Deoxythymidine kinase	dCDP	dTTP	Non-Linear	+, ?	Okazaki & Kornberg(1964) Bresnick et al. (1966)
Fructose-1,6-diphosphatase	?	AMP	Non-Linear	+, ?	Rosen et al. (1967)
Glutamate dehydrogenase	ADP	GTP	Non-Linear	+,>10	Frieden & Coleman (1967)
Glutaminase	Succinate	Cl^-	Non-Linear	+, ?	Kvamme et al. (1965)
L-Glutamine-D-fructose-6-phosphate transaminase	?	UDP-N-Acetyl glucosamine	Linear	?	Kornfeld (1967)
Glutamine phosphoribosylpyrophosphate amidotransferase	?	GMP,AMP	Non-Linear	?	Nierlick & Magasanik (1965)

TABLE 1. : cont'd.

Enzyme	Activator	Inhibitor	Kinetic Properties[c]	Subunit Structure[d]	Reference
Glyceraldehyde-3-phosphate dehydrogenase	?	ATP	Non-Linear	+, 4	Kirschner et al.(1966)
Glycogen synthetase	Glucose-6-P	UDP	Non-Linear	?	Rothman & Cabib (1967)
Guanine amino-hydrolase	GTP	?	Non-Linear	?	Josan & Krishnan (1968)
Homoserine dehydrogenase	Isoleucine	Threonine	Non-Linear	+, ?	Datta et al. (1964)
IMP dehydro-genase	?	GMP	Non-Linear	?	Buzzee & Levin (1968)
Inorganic pyro-phosphatase	Mg^{2+}	$MgADP^-$	Non-Linear	?	Horn et al. (1967)
Isocitrate dehydrogenase (Neurospora)	AMP	?	Non-Linear	?	Sanwal & Cook (1966)
Isocitrate dehydrogenase (Mammalian)	ADP	TPNH	Non-Linear	?	Stein et al. (1967)
α-Isopropylmalate synthetase	Acetyl-CoA	Leucine	Linear	+, 3	Webster et al. (1965)
Methylene tetra-hydrofolate reductase	?	S-Adenosyl-methionine	Linear	?	Kutzbach & Stokstad (1967)
NADH oxidase	AMP	?	Non-Linear	?	Worcel et al. (1965)

TABLE 1. : cont'd.

Enzyme	Activator	Inhibitor	Kinetic Properties[c]	Subunit Structure[d]	Reference
Nucleoside diphosphatase	$MgATP^{2-}$	?	Non-Linear	?	Yamazaki & Hayaishi (1968) Schramm & Morrison (1968)
Phosphofructokinase	AMP	ATP	Non-Linear	+,>10	Lindell & Stellwagen (1968)
Phosphoglycerate dehydrogenase	?	Serine	Non-Linear	+, 4	Sugimoto & Pizer (1968a,b) Rosenbloom et al. (1968)
Phosphoribosyl-ATP-pyrophosphorylase	?	Histidine	Linear	+, ?	Martin (1963)
Phosphoribulokinase	?	AMP	Non-Linear	?	MacElroy et al. (1968)
Phosphorylase a	AMP	?	Non-Linear	+, 4	Morgan & Parmeggiani (1964)
Phosphorylase b	AMP	ATP	Non-Linear	+, 2	Helmreich & Cori (1964)
Pyruvate kinase	AMP	Fructose-1,6-diphosphate	Non-Linear	+, 4	Maeba & Sanwal (1968) Steinmetz & Deal (1966)
Pyruvate carboxylase	Acetyl-CoA	?	Non-Linear	+, 4	Scrutton & Utter (1967)
Ribonucleotide diphosphate reductase	ATP,dTTP	dATP	Non-Linear	?	Larsson & Reichard (1966)
Ribonucleotide reductase	dTTP,dATP, dCTP,dGTP	?	Non-Linear	?	Beck (1967)

TABLE 1. : cont'd.

Enzyme	Activator	Inhibitor	Kinetic Properties[c]	Subunit Structure[d]	Reference
dTDP-D-Glucose pyrophosphorylase	?	dTDP-L-rhamnose	Non-Linear	?	Melo & Glaser (1965)
Threonine deaminase	Valine	Isoleucine	Non-Linear	+, 4	Zarlengo et al. (1968)
Trehalose synthetase	?	Trehalose	Non-Linear	?	Murphy & Wyatt (1965)
Tryptophan pyrrolase	Ascorbate	NADPH	Non-Linear	+,>10	Cho-Chung & Pitot (1967)
UDP-D-Glucose pyrophosphorylase	?	AMP	Non-Linear	?	Kornfield (1965)
UDP-Glucose dehydrogenase	?	UDP-D-xylose	Non-Linear	?	Ankel et al. (1966)
UDP-N-acetyl-glucosamine epimerase	?	CMP-N-acetyl neuramine acid	Non-Linear	?	Kornfield et al. (1964)

[a]Due to the plethora of literature in this field, it is possible that omissions may be found in the table. The survey was completed in July, 1968.

[b]Question marks indicate that no knowledge pertaining to the trait described in that column have been published.

[c]Kinetic properties are in reference to double reciprocal plots of initial velocity against the concentration of any reactant (substrate, activator or inhibitor) of the enzyme.

[d]A + indicates that the enzyme has been made to appear in more than one polymeric form. A number in this column indicates the number of observed subunits which may or may not be composed of more than one polypeptide chain.

Criteria for classifying enzymes as being allosteric

Originally, the term allosteric, as described by Monod and Jacob (1961), was reserved for the situation whereby reactants combined at a site on an enzyme which was different from the stereospecific site at which substrate combines and undergoes reaction, i.e. the catalytic site. This is implied by the prefix allo-, which is derived from the Greek allos, "a combination form denoting differentiation from the normal..." (Webster's New Collegiate Dictionary, 1953). Soon after this definition was proposed, its original meaning was ignored by including such proteins as hemoglobin in the allosteric classification because of the sigmoidicity exhibited in the oxygen binding curve (Monod et al., 1965).

Recently the tendency has been to class as allosteric, all enzymes which show any form of activation or inhibition that may be of physiological significance (cf. Stadtman, 1966). So, despite the relative specificity of the original definition, the emphasis has shifted to a much broader and less well defined one which is largely dependent on metabolic significance.

This broad definition raises many problems with

respect to the types of inhibition encountered. Thus from studies of enzymes exhibiting classical Michaelis kinetics it has been shown that enzyme inhibition may occur as a result of the addition of single reaction products, or by dead-end inhibitors, which cause non-reactant enzyme forms to accumulate. Further, enzymes may be inhibited by compounds which are so closely related to the substrates or products that they combine at the same stereospecific sites. Under the present methods for defining allosteric interactions, inhibitions arising from reactions of the above type are likely to be termed allosteric if they could be of some physiological significance. While it must be admitted that there are difficulties associated with the classification of allosteric enzymes, the inclusion of enzymes which are so inhibited achieves little that is unique and adds much to the confusion that surrounds the definition of an allosteric enzyme.

One definite criterion for allosterism, as defined by Monod and Jacob (1961), is the ability of the allosteric properties of an enzyme to be destroyed by treatment such as heat or mercurials without destroying the catalytic properties. A result of this type would seem to indicate that the catalytic and

allosteric properties are independent of one another. However, it must be considered that if overlapping or interacting substrate and allosteric sites did exist, destruction of one site would not necessarily destroy the other. It has not been established that all, or even a large number of enzymes which may be considered to be allosteric can be characterized in this way. It may well be that enzymes with the above properties belong to a special class of allosteric enzymes.

As physiological significance of allosteric effects has been considered in classing enzymes as allosteric, it might be concluded that the *in vivo* effects should play a role in allosteric classification. However, *in vitro* studies do not necessarily relate to *in vivo* effects, even though the very low concentrations of allosteric modifiers needed to affect activity do suggest a metabolic role. As the *in vivo* significance of most allosteric effects are difficult to assess, such a criterion should not be used in the classification of enzymes as being allosteric.

In order to compile the list of allosteric enzymes as given in Table 1, it was necessary to have in mind criteria for deciding if particular enzymes could be classified as being allosteric. Enzymes were

considered to be allosteric if they : (a) were activated or inhibited by a compound which was not a substrate or product of the reaction; and (b) exhibited at least one sigmoidal plot of initial velocity against reactant concentration, where reactant may be either a substrate or allosteric modifier. In the cases where all plots of initial velocity against reactant concentration were hyperbolic, the enzymes were considered to be allosteric only if the allosteric properties could be destroyed without destroying the catalytic properties (e.g. L-glutamine-D-fructose-6-phosphate transaminase, Kornfeld, 1967).

Function of allosteric enzymes

The function of allosteric enzymes is presumably to regulate the steady-state concentration of reactants which are available for a metabolic pathway, and in doing so, maintain the intricate chemical balance which is required for any organism to function efficiently.

The rate at which an allosteric enzyme catalyzes its reaction may be affected by either the levels of substrate present or by the presence of allosteric modifiers. Control by substrate concentration results from the fact that over certain ranges, a small increase or decrease in substrate concentration results

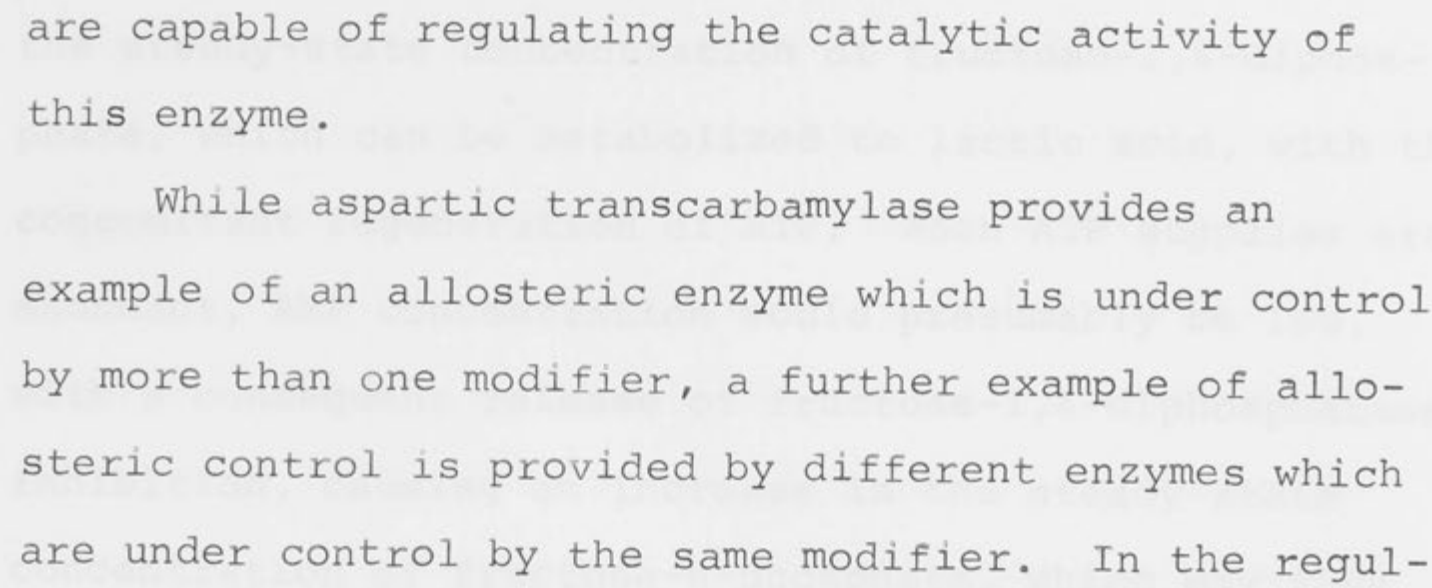
in a marked increase or decrease in catalytic activity. It is this property of allosteric enzymes that gives rise to the often observed sigmoidal initial velocity curves. Regulation of these enzyme activities may also occur by the presence of allosteric modifiers which activate or inhibit the catalytic activity of the enzyme.

These types of control are an advantage over regulation of activity by induction or repression of enzyme synthesis, as allosteric controls are immediate, whereas a considerable time lag occurs before activity may be regulated by induction or repression.

An example of an enzyme that is under control by both allosteric activation and inhibition, is aspartic transcarbamylase, which catalyzes the first unique step in the pathway leading to the synthesis of pyrimidine nucleotides. This reaction is inhibited by the ultimate product of the pathway, CTP, whose formation proceeds by way of UTP. The purpose of CTP inhibition is presumably to prevent overproduction of pyrimidine nucleoside triphosphates along this pathway. Conversely, the purine nucleotide, ATP, activates aspartic transcarbamylase. Thus in order to maintain the balance of purine and pyrimidine nucleotides needed for nucleic acid synthesis, representatives of both the

purine and pyrimidine nucleotide pools, ATP and CTP, are capable of regulating the catalytic activity of this enzyme.

While aspartic transcarbamylase provides an example of an allosteric enzyme which is under control by more than one modifier, a further example of allosteric control is provided by different enzymes which are under control by the same modifier. In the regulation of glycolysis and gluconeogenesis AMP is thought to play a major role by acting as an activator of phosphofructokinase and as an inhibitor of fructose-1,6-diphosphatase. As these two enzymes may be rate controlling for glycolysis and gluconeogenesis (Rosen et al., 1967), the levels of AMP are able to determine the relative rates of these processes.



Thus when cellular energy supplies are low, the high AMP concentrations present under these conditions would cause activation of phosphofructokinase and

inhibition of fructose-1,6-diphosphatase. This increases the steady-state concentration of fructose-1,6-diphosphate, which can be metabolized to lactic acid, with the concomitant regeneration of ATP. When ATP supplies are abundant, AMP concentration would presumably be low, with a consequent release of fructose-1,6-diphosphatase inhibition, causing an increase in the steady-state concentration of fructose-6-phosphate, which may then be used for glycogen synthesis. By this means, allosteric controls may play an important role in the energy balance of cellular metabolism. Although it is very difficult to demonstrate the metabolic significance of most allosteric enzymes *in vivo*, the above scheme does seem to operate in pigeon liver homogenates (Krebs, Newsholme, Speake, Gascoyne and Lund, 1964).

Theories which Attempt to Explain Allosteric Effects

Over the past ten years, investigations of many allosteric enzymes have been made, but these have been largely descriptive, and have dealt with the general properties of these enzymes (cf. the reviews of Stadtman (1966) and Atkinson (1966) for numerous examples).

Early speculation which attempted to explain the properties of allosteric enzymes, was based on the

hypothesis that allosteric enzymes contained multiple combining sites for substrates and modifiers that could be either overlapping or separate. Thus when saturating substrate concentrations could overcome the effect of the allosteric inhibitor, overlapping sites were proposed (Umbarger, 1961). On the other hand, when saturating substrate concentrations did not overcome this effect, or when the allosteric properties could be destroyed without destroying the catalytic activity, separate sites were posulated such that the modifier site could affect the properties of the catalytic site (Changeux, 1961). Although these assumptions could account for the observed effects, there was no further development of these theories at that time, and no experiments were devised to test their validity.

Other theories which could account for the experimental data with allosteric enzymes soon appeared, and these differed from the earlier theories of Umbarger (1961) and Changeux (1961) in that they were based solely on thermodynamic effects that related to isomerization, polymerization, and interaction of subunits as affected by multiple bindings of substrate and modifier molecules (Monod, Wyman and Changeux, 1965; Koshland, Nemethy and Filmer, 1966; Nichol,

Jackson and Winzor, 1967). Thus over the past seven years two types of theory have been developed to explain allosteric action; one based on effects between enzyme and modifier sites on an enzyme, and one based on thermodynamic interactions which occur between identical subunits of the allosteric enzyme molecule.

The following sections of the Introduction will discuss the development of these different theories to explain allosteric action under the subheadings of : (1) Kinetic theory for allosteric enzymes, (2) The cooperativity theory of Monod, Wyman and Changeux (1965), (3) The sequential theory of Koshland, Nemethy and Filmer (1966), and (4) The polymerization theory of Nichol, Jackson and Winzor (1967).

Kinetic theory for allosteric enzymes

Even before allosteric enzymes were discovered, it had been noted that certain substances caused activation or inhibition of enzymes, and that many enzymes gave initial velocity plots which deviated from the Michaelis-Menten (1913) formulation in that plots of initial velocity against substrate concentration were sigmoidal. The simplest explanation of these sigmoidal plots is that each enzyme molecule has several catalytic sites at which substrates may

combine in an interdependent manner (cf. Stadtman, 1966; Atkinson, 1966), i.e. that the combination of one substrate molecule affects the combination of the next, or changes the catalytic ability of the next catalytic site. The effects of activators and inhibitors were the subject of several theoretical papers (Botts, 1958; Dalziel, 1958) in which the authors derived initial velocity equations which could account for these types of kinetic data.

After the discovery of allosteric enzymes, attention was drawn to the fact (Frieden, 1964) that the kinetic data observed with allosteric enzymes could be accounted for in terms of classical kinetic theory. Frieden derived initial velocity equations which allowed the combination of allosteric modifiers to give enzyme-modifier forms which had properties different from those of the native enzyme. In this formulation, the various enzyme forms could show differences in affinity for any reactant, or could form products at different rates.

Assumptions which pertain to the subunit structure, degree of polymerization or conformational changes of the enzyme are not required for the theory, although such changes may result as a consequence of

reactant bindings. It must be pointed out however, that these changes in the physical nature of the enzyme cannot be detected by kinetic means.

Development of the cooperativity theory of Monod, Wyman and Changeux (1965)

The literature relating to allosteric enzymes was reviewed by Monod, Changeux and Jacob (1963) in order to find features which were common to all of these enzymes. From this study they concluded that :

a. No direct interactions of any kind need occur between the substrate and modifier binding sites of an allosteric protein.

b. Allosteric effects are due entirely to a reversible conformational alteration induced in the protein when it binds the substrates or modifiers.

These conclusions were based on reports that in some of the allosteric enzymes which had been studied, the allosteric properties could be destroyed, while at the same time the catalytic activity was unaffected or even enhanced by treatment such as heating or reaction with mercurials. Thus the sites which determined the allosteric properties were envisaged as entities which were divorced from the catalytic sites, and which

mediated their effects on the catalytic sites by conformational changes in the protein structure.

This theory was further developed by Monod, Wyman and Changeux (1965). It differed from the theory proposed in 1963 in assuming that allosteric proteins are composed of a number of identical subunits (protomers) which form the complete enzyme molecule (oligomer). (The nomenclature used in the physical description of the enzyme structure is illustrated in Figure 1). Allosteric effects in this model are classified as "homotropic" and "heterotropic". Any reactant combining in such a way as to give a sigmoidal binding curve would be termed as a homotropic interaction, while the effect of the combination of unlike reactants (i.e. substrate and modifier) would be termed a heterotropic interaction.

In order to explain how allosteric interactions may take place in this system, the following model has been proposed :

a. The protomers occupy equivalent positions in the oligomer. This gives the oligomer at least one axis of symmetry.

b. There is only one stereospecific site for each reactant which binds to the protomer. Thus for

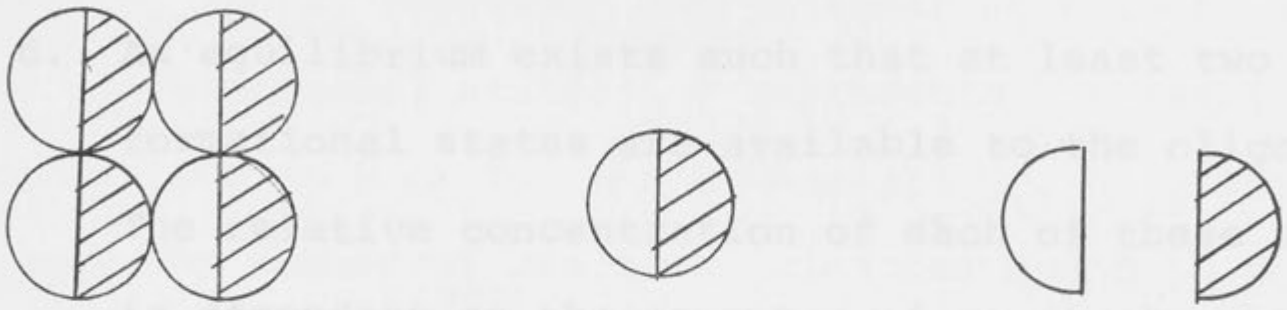
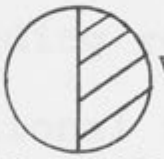


FIGURE 1

Where  and  are non-identical polypeptide chains. When in the oligomer,  would be referred to as a protomer. The same nomenclature would be applied when each protomer consisted of a single polypeptide chain. In that case, the monomers and subunits would be identical.

an enzyme which has one substrate and one modifier there would be two stereospecific sites per protomer.

c. The conformation of each protomer is constrained by the bonds which exist between protomers (inter-protomer bonding).

d. An equilibrium exists such that at least two conformational states are available to the oligomer. The relative concentration of each of these states is dependent on the presence of reactants which bind to the protomer(s) and thereby shift the equilibrium between these two conformational states.

e. The affinity of one or more of the stereospecific sites towards its corresponding reactant is altered when this conformational transition takes place.

f. The oligomer maintains its molecular symmetry in both conformational states, thus all protomers must change their conformations simultaneously.

The binding sequence for this model is written :

$$R_o \rightleftharpoons T_o$$

$$R_o + F \rightleftharpoons R_1 \qquad T_o + F \rightleftharpoons T_1$$

$$R_1 + F \rightleftharpoons R_2 \qquad T_1 + F \rightleftharpoons T_2$$

$$R_{n-1} + F \rightleftharpoons R_n \qquad T_{n-1} + F \rightleftharpoons T_n$$

where R_o and T_o represent the oligomer in each of its two conformational states; F represents the reactant which binds to R or T; the subscripts $_{o,1,...n}$ represent the number of reactant molecules bound to the oligomer, and n represents the number of protomers in the oligomer. The multiple bindings of a specific reactant to all R forms of the enzyme takes place with equal affinity, and likewise a reactant binds to all T forms of the enzyme with equal affinity. Thus within either molecular entity (R_o or T_o) all binding sites for a specific reactant are independent. Because of this, reactant binding to only one conformational state (R_o or T_o) would give rectangular hyperbolic (Michaelis type) binding curves. If both R_o and T_o conformational states bound the reactant with equal affinity, they would be equivalent and again Michaelis type binding curves would be obtained. Sigmoidal binding curves would be exhibited when the equilibrium

between R_o and T_o is such that the form of enzyme which binds reactants poorly is present at much higher concentrations than the form of enzyme which binds reactants with higher affinity.

The effect of heterotropic reactants (allosteric modifiers) is to shift the equilibrium between the R and T conformational forms as a consequence of binding to one or other of these forms. Thus activators would bind to the form which had the highest affinity for substrate, thereby shifting the $R \rightleftharpoons T$ equilibrium in the direction which favours substrate binding. Allosteric inhibitors would exert their effects in a similar but opposite manner.

In deriving the equations that describe this system, the assumption is made that in either conformational state (R_o or T_o) all binding sites are equivalent and that combination of one substrate molecule cannot affect the binding of another in that conformational state. As no allowance is made for the rate of breakdown to give products, the theory of Monod et al. (1965) may not be used to interpret initial velocity data unless the following assumptions are made : (a) the addition and release of all substrate molecules and the transformations between

conformational states are very fast compared to the breakdown of substrates to form products; (b) allosteric modifiers cannot change the substrate affinity or the maximum velocity of the reaction, and can act only by changing the ratio of the two conformational states; and (c) the equations can be used only to define substrate effects rather than modifier effects (cf. Frieden, 1967). As assumption (a) has been shown to be false for a number of non-allosteric enzymes (e.g. Hsu, Cleland and Anderson, 1966; Bridger and Cohen, 1968; Soldin and Balinsky, 1968) there is no a priori reason to assume that it would be true for all allosteric enzymes. Thus, it would appear that while thermodynamic data may be interpreted by this model, there is little justification for interpreting kinetic data according to this model.

The sequential theory of Koshland, Nemethy and Filmer (1966); Kirtley and Koshland (1967) and Haber and Koshland (1967)

This theory was developed in order to provide a model for allosteric enzyme action which was less restrictive than that proposed by Monod et al. (1965) with respect to the number of different enzyme species that could be formed. Thus, it is based on the

postulates that (a) allosteric enzymes are composed of a number of identical subunits; (b) each subunit may exist in at least two conformational states; (c) a change in conformation of one subunit may, or may not, change the stability of the conformations of neighbouring subunits by subunit interactions; and (d) the reactants bind preferentially to one of the conformational states.

The manner in which reactants combine with the enzyme has been considered for an enzyme containing four identical subunits arranged in a "square" configuration (Kirtley and Koshland, 1967; Haber and Koshland, 1967). Other geometrical arrangements for four subunits have been considered by Koshland et al. (1966), and include "linear" and "tetrahedral" arrangements which are possible for four subunits and lead to different forms of subunit interactions. Thus in the square arrangement, each subunit could interact with two neighbouring subunits; in a linear arrangement the end subunits could interact with only one subunit, while the middle two subunits could each interact with two subunits; and in a tetrahedral arrangement each subunit could interact with all three neighbouring subunits. It has been shown (Koshland

et al., 1966) that the experimental data which would result from these different geometrical arrangements cannot be differentiated by any known method. Therefore, only the square case has been considered in the theoretical treatment which includes both substrates and allosteric modifiers as reactants (Kirtley and Koshland, 1967).

In order to explain sigmoidal substrate binding curves, the Kirtley and Koshland (1967) theory proposes that each subunit of the enzyme may exist in two or more conformational states, and when substrate binds, this subunit changes its conformational state. Further, the subunit in the new conformational state may affect the neighbouring subunits such that they now have an altered affinity for the substrate. Figure 2.A illustrates the different possible ways that this conformational change may take place for an individual subunit. However, as these different pathways leading to the new conformational state are assumed to be in an equilibrium situation, thermodynamic considerations require that formation of the new state by any pathway gives the same overall equilibrium constant. Following the conformational change in the first subunit, the neighbouring subunits may or may not be induced to

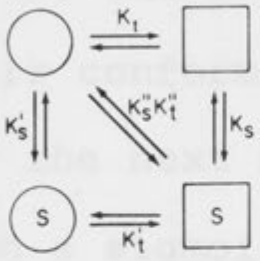


FIGURE 2.A.

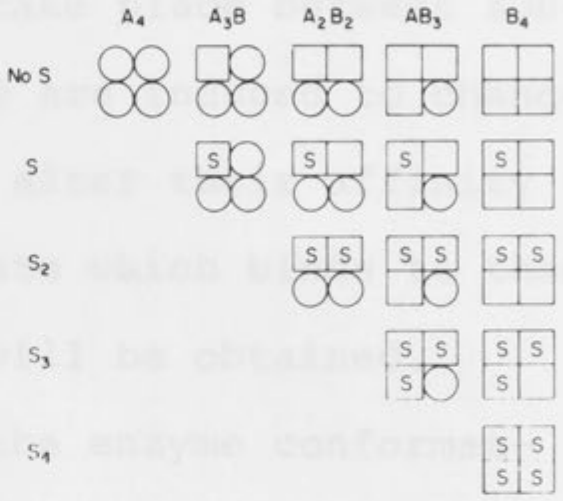


FIGURE 2.B.

(Adopted from Haber and Koshland, 1967)

Different pathways by which a subunit conformational change may take place. □ and O represent different conformations and S represents the substrate. Because of thermodynamic considerations, $K''_sK''_t = K_sK_t = K'_sK'_t$.

Possible enzyme-substrate forms for an enzyme of four subunits when substrate only is bound, and when the subunits may exist in only two conformational states.

change their conformational states by virtue of interactions which occur between neighbouring subunits. If no interactions are mediated between neighbouring subunits, each one may be considered to be independent, and hyperbolic binding curves will be obtained. On the other hand, if interactions do take place between subunits, and neighbouring subunits are induced to change their conformations and thereby alter their affinity for the next molecule of substrate which binds to them, then a sigmoidal binding curve will be obtained. Figure 2.B illustrates some of the enzyme conformational forms and enzyme-substrate complexes which can result from the situation described above.

The relationships between substrate and allosteric modifier binding to the enzyme are best seen by use of the diagrams from Kirtley and Koshland (1967), which are reproduced in Figure 3. The reactants X and L, where X is substrate and L is the allosteric modifier, may both combine with every subunit, and several possible modes of their combination are considered. Thus when L combines to produce a conformational form with increased or decreased affinity for substrate (X) it acts as an allosteric activator and inhibitor, respectively.

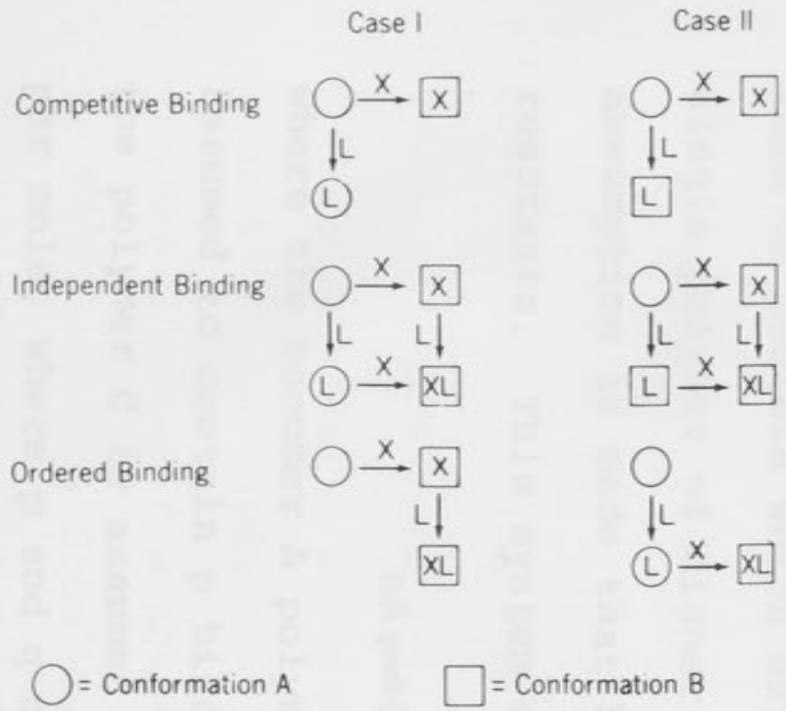


FIGURE 3.A.

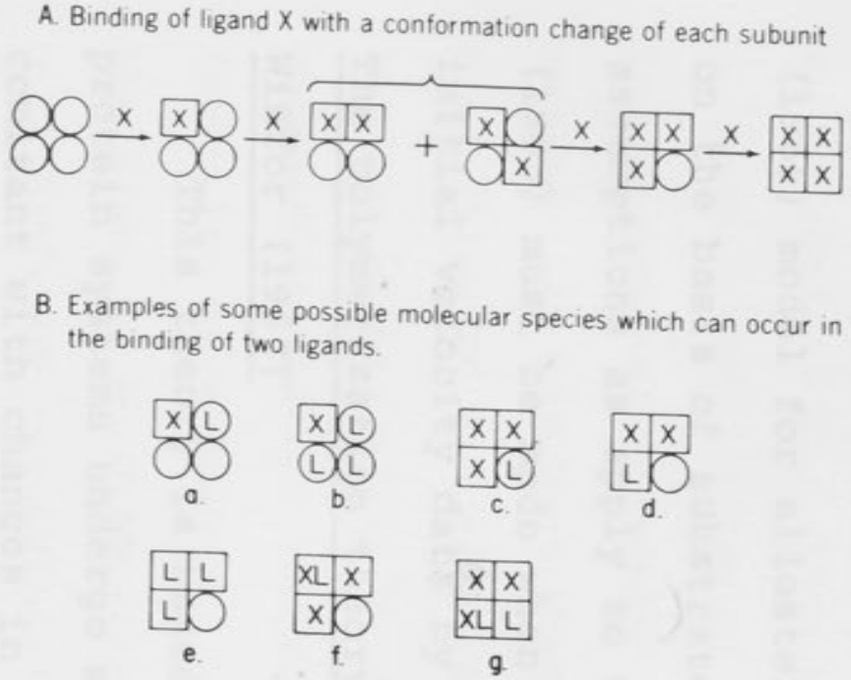


FIGURE 3.B.

(Adopted from Kirtley and Koshland, 1967)

Examples of the types of binding that may occur on a single subunit with two reactants (X and L) present. In the enzyme molecule, this type of binding would occur on each of the four subunits. The modifier L may or may not cause a conformational change (from A to B) while reactant X must always cause a conformational change.

Examples of the molecular species which can result when only X or when X and L bind to the enzyme. When a subunit changes from the A to the B conformation (○ to □), neighbouring subunits may or may not be affected. When X and L are bound to the same subunit, the conformation is the same as when X alone is bound.

The equations that describe the Koshland et al. (1966) model for allosteric effects have been derived on the basis of substrate binding. Therefore similar assumptions as apply to the theory of Monod et al. (1965) must be made when attempting to interpret initial velocity data by this theory.

The polymerization theory of Nichol, Jackson and Winzor (1967)

This theory is based on the observation that many protein systems undergo molecular weight changes concomitant with changes in protein concentration (cf. Nichol, 1965). Thus, it differs from those discussed above in that it was developed in order to explain allosteric effects by the existence of a monomeric form of protein which exists in equilibrium with a single polymer of higher molecular weight. The assumption is made that both species may bind the reactants. This system may be represented as :

$$nA \rightleftharpoons C$$

where the monomer A polymerizes to give C. A is assumed to contain p binding sites per mole, while the polymer C is assumed to contain q binding sites per mole, where p and q may or may not be equal.

However, all binding sites within A or C are considered to be equivalent. Thus when np = q, and the binding sites on both A and C forms of enzyme have equal affinity for reactants, Michaelis binding curves will be obtained. In the case where np ≠ q, or the binding properties of the sites on A and C differ, sigmoidal binding curves would be obtained. Binding equations which describe the various methods of polymeric formation have been derived and used to illustrate the types of binding curves which may be obtained.

Although the authors have not considered the action of allosteric modifiers, they could presumably act by preferential binding to a site(s) on one or another of the monomeric or polymeric forms, thereby shifting the equilibrium of polymerization.

Studies of Allosteric Reaction Mechanisms

Experimental investigations which have made a contribution to the understanding of allosteric reaction mechanisms will be described in this section.

Quantitative analysis of kinetic data from allosteric enzyme investigations has rarely been attempted, with the notable exceptions of the work on $NADH_2$ oxidase from Mycobacterium tuberculosis by Worcel, Goldman and Cleland (1965), and the work

on the NAD specific isocitric dehydrogenase from Neurospora crassa by Sanwal, Stachow and Cook (1965) and Sanwal and Cook (1966). In these instances the kinetic data were analyzed according to the initial velocity equations and methods of numerical analysis which had been developed by Cleland (1963a,b,c,d).

NAD specific isocitrate dehydrogenase. It was concluded that the results obtained by analyzing the data for the NAD specific isocitric dehydrogenase reaction were consistent with a reaction mechanism that changed from ordered to random in the presence of the allosteric modifier, AMP. Further, the conclusions were reached that the enzyme possesses two sites at which either the substrate, isocitrate, or the allosteric activator, citrate, could combine and that it was essential for one site to react with either citrate or isocitrate before reaction could occur at the second site.

$NADH_2$ oxidase. The analysis of kinetic data for the $NADH_2$ oxidase reaction (Worcel et al., 1965) was consistent with the enzyme containing two inter-dependent sites for combination of $NADH_2$. Double reciprocal plots of initial velocity (v) against $NADH_2$ were therefore non-linear. When high concen-

trations of the allosteric activator, AMP, were added, plots of 1/v against $1/NADH_2$ became linear. Further, plots of v against AMP in the presence of $NADH_2$ were hyperbolic, indicating only one binding site for AMP. These results were interpreted to mean that in the absence of AMP the enzyme has two sites for $NADH_2$ combination, both of which must be filled before reaction can occur. When AMP is present, it combines at one of these sites, leaving the second site for $NADH_2$ combination. Thus when AMP is present at high concentrations, only one molecule of AMP and one molecule of $NADH_2$ combine with the enzyme.

Further investigations showed that the activation of the enzyme by AMP was a rapid reaction and involved a conformational change. Thus it was demonstrated that when AMP was removed by passage of a solution, containing both AMP and enzyme, through a column of G-25 Sephadex, the enzyme was obtained in its activated form which only reverted slowly to its less active native state.

Aspartate transcarbamylase was one of the first allosteric enzymes to be described (Yates and Pardee, 1956), and has been more thoroughly studied by thermodynamic methods than any other allosteric enzyme. The

recent studies of Changeux, Gerhart and Schachman (1968), Gerhart and Schachman (1968), Changeux and Rubin (1968) and Weber (1968a) have taken advantage of the fact that the native enzyme may be separated into subunits after treatment with p-mercuribenzoate to show that it is composed of four identical regulatory subunits and two identical catalytic subunits. The regulatory subunits were found to consist of a single polypeptide chain, each of which bound one molecule of modifier, while the catalytic subunits consisted of two polypeptide chains and bound two molecules of each substrate, carbamylphosphate and succinate (a competitive inhibitor with respect to aspartate). In accordance with the results, the native enzyme was found to bind four molecules of allosteric modifier and four sets of substrate molecules. From these data the conclusion was reached that aspartate transcarbamylase exists as a tetramer of four identical monomers, each of which contains one regulatory and a catalytic polypeptide chain (Changeux et al., 1968).

Binding studies carried out using carbamylphosphate and succinate (in place of aspartate) demonstrated that these two compounds combined in an

ordered manner, such that carbamylphosphate must be present before succinate may be bound to the enzyme. Further studies showed that a sigmoidal substrate binding curve was obtained with the native enzyme, but not with the isolated catalytic subunit. The fact that small but significant changes in sedimentation rates occurred with the binding of substrates to the native enzyme, but not the catalytic subunits, suggested that the observed sigmoidal binding curves were a result of enzyme conformational changes. By equating these changes in sedimentation rate with an $R \rightleftharpoons T$ conformational change, Changeux and Rubin (1968) have concluded that these data are consistent with the allosteric theory of Monod et al. (1965). However, the same authors pointed out that this was not the sole explanation which could account for the experimental results.

Recent amino acid sequence and X-ray crystallographic studies on aspartate transcarbamylase suggest that the molecule may exist not as a tetramer, with each monomer consisting of one catalytic and one regulatory polypeptide chain, but rather as a trimer with each monomer being composed of two regulatory peptide chains and two catalytic peptide chains

(Weber, 1968b; Wiley and Lipscomb, 1968). Thus the subunit structure of the enzyme must now be regarded as being uncertain.

Investigations have also been carried out to determine what effects substrates, substrate analogues and allosteric modifiers have on the rates of proteolysis of aspartate transcarbamylase (McClintock and Markus, 1968). From the results of this study it appears that aspartate does bind to the enzyme in the absence of carbamylphosphate and that the analogue of aspartate, succinate, does not act in the same manner as aspartate. Therefore the results of the binding studies carried out by using succinate in place of aspartate became of questionable validity. The results obtained by measuring the rates of digestion in the presence of either the allosteric activator, ATP, or aspartate indicated that these two reactants do not combine with the same conformational form of the enzyme as required by the Monod et al. (1965) model.

The above section of the Introduction has illustrated the attempts to interpret the experimental results obtained with allosteric enzymes. The examples given have been the ones which are most

informative with respect to the elucidation of allosteric reaction mechanism, and yet, very little definitive information has been gained. Further, the results of these investigations cannot be used to distinguish between the various theories which have been proposed to account for allosteric effects.

Determination of Allosteric Reaction Mechanisms

In order to understand any allosteric reaction mechanism, it will be necessary to know the nature of enzyme-substrate interactions and the effects of allosteric modifiers upon these interactions.

Problems that must be solved with respect to enzyme-substrate interactions are : (1) the number of substrate molecules which react per molecule of enzyme; (2) the number of substrate molecules that react in an interdependent manner; and (3) the factors which cause interdependent substrate reactions.

This section of the Introduction will discuss experimental approaches to the above problems and will point out the difficulties and shortcomings of these techniques that may be used to investigate allosteric interactions.

The study of enzyme-substrate interactions

Determining the number of substrate binding sites per enzyme molecule. In order to answer the question of how many moles of substrate react per mole of enzyme, thermodynamic experiments such as equilibrium dialysis or observation of spectral changes which occur upon the binding of substrate (or substrate analogue) molecules must be carried out over a range of substrate concentrations. Plotting these data by the method of Scatchard (1949) will give the total number of substrate moles bound per mole of enzyme. This answer is achieved irrespective of whether the substrate bindings are independent or interdependent, although the form of the plot will be different for the two situations.

Although such experiments appear feasible, difficulties arise because : large quantities of pure enzyme of known molecular weight are needed for such an analysis; non-specific substrate bindings will give an erroneously high number of binding sites; the binding experiments are very time consuming; the data from equilibrium dialysis studies are of low accuracy, especially in the critical regions of high substrate concentration; and few allosteric enzymes

exhibit spectral changes upon substrate binding.

Because of the above difficulties, binding data have rarely been used to determine the number of binding sites. Instead, a substitute method which is referred to as a Hill plot and which employs initial velocity data to determine the number of binding sites has been used (cf. Brown and Hill, 1922). However, the number of binding sites obtained from analysis of data by the Hill plot will not be correct unless the following assumptions are met : all active sites must be interdependent, the rate of product formation is very slow compared with the rate of substrate addition and release, and that the only enzyme-substrate form present in kinetically significant amounts is fully saturated with substrate molecules. As it is difficult to ascertain if these assumptions are true, and because there is no a priori reason why they should be true, such results must be interpreted with extreme caution, and can never be the sole basis for assigning a number of substrate sites to a particular enzyme, as is attested by the frequently observed non-integral values for the number of binding sites. Indeed, the use of the Hill plot has changed from being a

method for estimating binding sites (e.g. Atkinson et al., 1965) to one which defines a factor termed the "interaction factor" (Changeux, 1963).

Determining the number of substrate molecules which react in an interdependent manner. The same thermodynamic data which was used for determining the number of binding sites could be used to determine the number of substrate molecules which bind in an interdependent manner. This number, may or may not be equal to the total number of binding sites. In order to determine the number it would be necessary to derive the theoretical binding equations which describe the interdependent binding of 2, 3, 4...n molecules of substrate. The data could then be quantitatively analyzed by each of these equations in turn, and the one giving the best fit to the data would determine the number of interacting sites. This analysis would also give values for the thermodynamic constants associated with the binding of each substrate molecule.

Although an analysis of this kind is theoretically possible, mathematical methods for such fitting of data have been developed only for mechanisms containing two interacting sites. Further, the

experimental data for such an analysis must be both extensive and accurate (see Chapter II), and from the published results of binding studies (Changeux et al., 1968) it would appear that the data are much too inaccurate for analysis of even the simplest case, which would be that where two interacting substrate reactions occur. Even if quantitative agreement is found between a binding equation and the experimental data, the interaction of a larger number of sites cannot be excluded unless it has been shown that the total number of binding sites equals the number of interacting substrate reactions. This is because interdependent substrate reactions do not manifest themselves unless the dissociation constant for each substrate combination is sufficiently different from the subsequent combinations.

Initial velocity data may also be used to determine the number of interdependent substrate interactions. This may be done in a manner similar to that described above for thermodynamic data, except that in deriving initial velocity equations it becomes necessary to make allowances not only for binding effects, but also for kinetic effects, i.e. that the various enzyme-substrate complexes may give

rise to products at different rates. Kinetic studies have the advantages of requiring small amounts of enzyme, and the results being much more accurate than most thermodynamic measurements. In the analysis of kinetic data, the same limitations apply with respect to the quantitative analysis of the experimental data.

Because of the difficulties in analyzing experimental data according to equations which describe interdependent reactions, the thermodynamic data from aspartate transcarbamylase have been analyzed by Changeux and Rubin (1968) by assuming that they can be fitted to an equation which describes the Monod et al. (1965) theory. Initial estimates of the values for the thermodynamic constants were obtained from plots of the data, and these estimates were varied until the theoretical line described by the equation gave what appeared to be a good fit to the data. While constants have been obtained by this method, it must be pointed out that this type of analysis does not demonstrate that the data cannot be fitted equally well by other equations. The constants so obtained cannot be considered valid since the best fit to the data was not obtained by any procedure involving statistical methods. Under this circumstance there

can be more than one unique combination of values that appear to fit the data equally well.

In order to be able to readily determine the number of interacting substrate reactions, an extension to the present knowledge of curve fitting techniques for complex thermodynamic and initial velocity equations will be needed.

Factors which cause interdependent substrate reactions. The investigation of factors which cause interdependent substrate reactions is the most difficult part of an investigation into allosteric reaction mechanisms. This one aspect of allosteric reaction mechanisms has received extensive theoretical treatment (Monod et al., 1965; Koshland et al., 1966; Nichol et al., 1967) and interdependency effects have been attributed to subunit interactions, isomerizations or monomer-polymer reactions which affect only substrate binding. It should be noted that interdependency effects as observed by initial velocity measurements may be due to different rates of product formation from the various enzyme-substrate complexes of an ordered reaction, even if all substrate molecules combine with equal affinity. Such a proposal does not necessarily require conformational changes to take

place.

In order to determine if conformational changes take place in the enzyme upon substrate binding, it is necessary to study the physical form of the enzyme in the presence and absence of substrates (or substrate analogues) by a technique which can detect small changes in molecular shape. Such techniques are ultracentrifugation, proteolysis, optical rotary dispersion, and studies of the rates of enzyme reaction with reagents that combine with specific groups on the enzyme. As yet, these techniques have been used very little in the study of allosteric enzymes. Even when such techniques are used, there are difficulties associated with the detection of small conformational changes, as might be expected upon substrate combination. These relate to the large amounts of protein needed, the facts that the magnitude of the conformational change may be small and that the observed conformational change may not be associated with the interdependent substrate combinations. Particularly because of the latter problem, the results of studies which show conformational changes must be interpreted with caution.

In the case where all the molecules of one sub-

strate combine with equal affinity, any interdependency effect would be seen only by initial velocity studies and such a result must then be due to differences in the rate of product formation from different enzyme-substrate complexes. When the above situation is the case, a Scatchard (1949) plot of binding data would be linear, indicating independent binding sites, while a double reciprocal plot of initial velocity against substrate concentration would be non-linear.

Aspartate transcarbamylase provides an example for the use of techniques in order to determine conformational changes. This enzyme has been studied by ultracentrifugation (Gerhart and Schachman, 1968) and proteolysis (McClintock and Markus, 1968) techniques in order to determine the nature of the conformational changes, and although such changes were observed upon substrate binding, the two methods gave conflicting results for the manner in which the substrate bindings and conformational changes are related. Thus from one study (Gerhart and Schachman, 1968) it appeared that conformational changes occurred according to the proposal of Monod *et al*. (1965), while from the other study (McClintock and Markus, 1968) it appeared that the changes could be explained in terms

of the proposal of Koshland et al. (1966). Additional studies will be needed in order to clarify the nature of these conformational changes.

One factor that must be stressed in regard to enzyme conformational changes, is that kinetic investigations can give no indication of the nature of the various enzyme-substrate complexes (Morrison, 1965). Thus, no credibility can be given to results in which non-linear kinetic plots have been used as evidence for the existence of the enzyme in isomeric forms.

The study of the influence of allosteric modifiers

A complete understanding of allosteric reaction mechanisms also requires that examination be made of the effects of allosteric modifiers, as their presence causes marked changes in both the kinetic and binding properties of the enzyme. The manner in which modifiers influence allosteric enzyme reactions may be tested by studies of : (1) the effects of modifiers on substrate bindings; (2) the effects of modifiers on the velocity of the catalytic reaction; and (3) the nature of the modifier site(s) as opposed to the catalytic site(s).

The effects of modifiers on substrate bindings. The postulate that modifiers influence allosteric

enzyme reactions as a result of affecting the binding of substrate is open to test by undertaking thermodynamic studies with respect to substrate, both in the presence and absence of allosteric modifiers. Thus by determination of both the total number of substrate molecules bound and the number of interdependent substrate combinations, along with the thermodynamic constants associated with substrate combinations, the effect of modifiers on substrate binding could be determined. This ideal situation is usually prevented by the same difficulties which are encountered when studying the thermodynamics of substrate combinations which have been discussed above (see pp.29-35).

The effects of modifiers on the velocity of the catalytic reaction. In order to determine if allosteric modifiers alter the rate of product formation, kinetic studies must be carried out. Kinetic studies may be done in the absence of modifier and compared with experiments in which allosteric modifier is present at fixed concentrations, as a fixed variable, or when it is varied in constant ratio with respect to substrate. From quantitative analysis of such studies, information can be gained as to whether the velocity of product formation from enzyme-modifier-

substrate complexes is the same or different from that of enzyme-substrate complexes. Such studies may indicate whether substrate and modifier molecules compete for the same combining site, or if they combine at distinct sites on the enzyme.

This type of investigation is the only method of determining kinetic effects, and its use is again limited by the inability to be able to carry out quantitative analysis of complex data.

The nature of the modifier site(s) as compared to the catalytic site(s). Inherent in the original definition of the term, allosteric, was the idea that the modifier site was distinct from the catalytic site. To determine if this were true for a particular allosteric enzyme, denaturating agents such as heat, mercurials or specific amino acid reactants may be used to destroy the modifier site(s) while leaving the catalytic site(s) unaffected. In this type of experiment, it is essential that modification of only the modifier site(s) takes place, since conclusions depend on the determination of catalytic activity. Thus, protection of the catalytic site(s) may be achieved by addition of relatively high concentrations of a substrate analogue or one substrate and one substrate

analogue, depending on the substrate requirements of the enzyme.

Such studies have been extensively used to investigate allosteric enzymes, but the interpretation of such results are not unambiguous. Experimental results may indicate that the catalytic site(s) is destroyed before any changes occur in the allosteric site(s), in which case no definitive information may be obtained.

How should allosteric investigations be made?

In recent years the emphasis in the field of allosteric investigation has been on the interpretation of data according to the models proposed to account for allosteric action on the basis of conformational changes. Thus, many investigators have assumed, rather than shown, that conformational changes occur in the enzyme as a consequence of substrate binding and speculated on how they take place. Frequently the conclusions have not borne any relationship to the experimental findings.

The main criticism which can be made in connection with the present investigations of allosteric reaction mechanisms is that kinetic data are frequently used to interpret thermodynamic theories. A way of avoiding the false premises that can arise

in such a situation would be to start with a general premise which assumed that allosteric effects could be due to both thermodynamic and kinetic effects and that conformational changes may or may not take place. From this general premise, the appropriate techniques could be used to prove or disprove certain portions of the general premise until a specific scheme could be worked out for that enzyme.

As was pointed out by Atkinson et al. (1965), "A first step in generalization may be made by attempting to devise for each of several regulatory enzymes the simplest set of assumptions that is capable of predicting the behaviour of that enzyme reasonably well. When a number of such models are available, their similarities and differences should be helpful in the construction of a scheme (or schemes) having broader applicability, but remaining closely in touch with experimental findings."

The study of nucleoside diphosphatase to be described in this thesis attempts to explain allosteric mechanism in terms of the "...simplest set of assumptions..." with the interpretation "...remaining closely in touch with experimental findings."

Nucleoside Diphosphatase

Distribution and properties

Nucleoside diphosphatase was discovered by a number of workers almost simultaneously (Strominger, Heppel and Maxwell, 1954; Plaut, 1955; Gregory, 1955 and Gibson, Ayengar and Sanadi, 1955) and shown to hydrolyze the terminal phosphoryl groups of IDP, UDP and GDP but not those of ADP or CDP. The enzyme has been detected in bovine, ovine and rat livers (Strominger *et al.*, 1954; Plaut, 1955; Gregory, 1955) and in porcine kidney (Gibson *et al.*, 1955). A study of the distribution of nucleoside diphosphatase was carried out by Novikoff and Heus (1963) who showed that it was present in rat liver and bovine adrenal cortex. They further demonstrated, by enzymatic staining techniques, that the nucleoside diphosphatase activity was located in the region of the endoplasmic reticulum. By studying the solubilization of the enzyme from the microsomal fraction of liver, they concluded that it was localized in the membranes of the endoplasmic reticulum rather than in the microsomes which are attached to the endoplasmic reticulum. Novikoff and Heus also demonstrated, by enzymatic staining techniques, that all steroid-producing

tissues tested : ovary, testis, placenta and adrenal cortex, contain nucleoside diphosphatase. However, it has not been possible to demonstrate its presence in extracts of rat, guinea pig, or bovine heart (Heppel, Strominger and Maxwell, 1959). Partially purified preparations of enzyme from different sources have been shown to have similar properties.

The substrate specificity of nucleoside diphosphatase is broad in that IDP, UDP, GDP (Plaut, 1955; Gibson et al., 1955), ribose-5-pyrophosphate (Heppel et al., 1959), thiamine pyrophosphate (Gregory, 1955; Yamazaki and Hayaishi, 1965) and dGDP (Yamazaki and Hayaishi, 1965), are hydrolyzed. However, no significant hydrolysis of ADP, CDP or any nucleoside mono- or triphosphate has been observed. A divalent metal ion is required for the catalytic activity. Mg^{2+}, Mn^{2+} and Ca^{2+} are the best activators, while Co^{2+} and Zn^{2+} are very poor substitutes, and Ba^{2+}, Sr^{2+} and Ni^{2+} are ineffective (Gregory, 1955; Yamazaki and Hayaishi, 1968). The real meaning of these results is unclear, as the metal ions and IDP were added in fixed concentrations rather than by calculating the amounts of each needed for a fixed concentration of the metal-IDP complex, which, under these conditions would

vary depending on the relative stability constants for the different metal-IDP complexes. This is especially important in view of the fact that the metal-IDP complex is the substrate for the reaction (Results, Chapter I).

A number of authors have reported the pH optima for the hydrolysis of IDP as being between pH 6.9 and 7.6 (Plaut, 1955; Heppel et al., 1959; Novikoff and Heus, 1963 and Yamazaki and Hayaishi, 1968). In contrast to these findings, the pH optimum for the hydrolysis of thiamine pyrophosphate is 9.0 (Yamazaki and Hayaishi, 1968), and this may account for the report by Novikoff and Heus (1963) that at pH 7.2, nucleoside diphosphatase does not hydrolyze thiamine pyrophosphate.

Nucleoside diphosphatase is inhibited by high (0.1 M) concentrations of fluoride (Plaut, 1955; Yamazaki and Hayaishi, 1968), p-mercuribenzoate (0.2 mM) and inorganic pyrophosphate (0.4 mM). Early reports indicated that high concentrations of nucleoside triphosphates acted as inhibitors (Plaut, 1955; Novikoff and Heus, 1963), but later it was shown that, at low concentrations, nucleoside triphosphates markedly increase the reaction rate,

especially at low substrate concentrations (Yamazaki and Hayaishi, 1965; 1968). None of the investigators realized that because of the high stability constant of magnesium nucleoside triphosphates compared to that of magnesium nucleoside diphosphate (73,000 and 4,000; O'Sullivan and Perrin, 1964), high concentrations of nucleoside triphosphates were causing inhibition by reducing the concentration of metal available for formation of metal-IDP, the substrate for the reaction. It was therefore not until Yamazaki and Hayaishi (1965) added small amounts of nucleoside triphosphates while keeping the free metal at high concentrations, that nucleoside triphosphates were recognized as activators of the reaction.

The first kinetic data from nucleoside diphosphatase was obtained by Yamazaki and Hayaishi (1965) who showed that with varying concentrations of IDP, the double reciprocal plot of initial velocity against substrate concentration was non-linear. In the presence of small concentrations of ATP (0.08 mM), which did not undergo any reaction, this plot became linear as a result of the increase in the initial velocities at low substrate concentrations. Thus, nucleoside diphosphatase appeared to be an allo-

steric enzyme.

Yamazaki and Hayaishi have obtained the most highly purified preparations of nucleoside diphosphatase. Originally they achieved a 575-fold purification of the enzyme from rat liver and subsequently Yamazaki and Hayaishi (1968) have described the purification of nucleoside diphosphatase to apparent homogeneity from bovine liver microsomes. The latter enzyme exhibits the same properties as nucleoside diphosphatases from other sources. It was shown to have a molecular weight of 100,000 by ultracentrifugation techniques.

Possible functions of nucleoside diphosphatase

As with most allosteric enzymes, a role in physiological control has been proposed for nucleoside diphosphatase. Plaut (1955) and Gibson *et al.* (1955) have suggested that this enzyme could act by hydrolyzing the nucleoside diphosphates that are a product of the phosphoenolpyruvate carboxykinase reaction :

oxaloacetate + ITP (or GTP) $\rightleftharpoons$ phosphoenolpyruvate + IDP (or GDP) + CO_2

and thereby shift the reaction in the direction of

phosphoenolpyruvate formation. The findings of Yamazaki and Hayaishi (1965,1968) that nucleoside triphosphates were activators of the reaction led them to postulate that when the levels of nucleoside triphosphates are high, this rate of shifting the phosphoenolpyruvate carboxykinase reaction would be enhanced. The resultant high steady-state concentration of phosphoenolpyruvate would then favour the formation of glycogen.

It must be noted that in the above postulate, no consideration has been given to the presence of nucleoside diphosphokinase. This enzyme could assume the same role in shifting the equilibrium, as the nucleoside diphosphates would be removed and converted back to the triphosphate with the formation of ~~a mono-~~ ADP ~~phosphate~~. This would have the effect of shifting the equilibrium in favour of phosphoenolpyruvate.

Aims of the Work Presented in This Thesis

The purpose of this thesis was to undertake an investigation into the reaction mechanism of nucleoside diphosphatase~~s~~. The ideal method of doing this would have been to combine kinetic and thermodynamic investigations in order to determine the manner in

which reactants combine with the enzyme and to determine the effect of allosteric modifiers on the enzyme. However, because of the time required to purify relatively small amounts of protein, it became apparent that it would not be feasible to prepare the large amounts of pure enzyme required for the above type of investigation. Further, much time was spent developing sensitive assay methods for both IMP and inorganic phosphate, the products of the reaction. Such work was necessary in connection with obtaining accurate kinetic data, both for initial velocity and product inhibition studies reported in this thesis.

Thus, as all but a few thermodynamic experiments were precluded, it was decided to undertake an intensive kinetic investigation of the nucleoside diphosphatase reaction and to determine what information could be obtained with respect to the reaction mechanism by carrying out quantitative analysis of the experimental data.

CHAPTER I

PURIFICATION AND GENERAL PROPERTIES OF NUCLEOSIDE DIPHOSPHATASE

CHAPTER I

PURIFICATION AND GENERAL PROPERTIES OF NUCLEOSIDE DIPHOSPHATASE

Introduction

Because of the interest of this laboratory in the mechanism of enzymes which catalyze phosphoryl group transfer reactions, the enzyme chosen for the study of an allosteric reaction mechanism was nucleoside diphosphatase. The allosteric properties of this enzyme were first reported by Yamazaki and Hayaishi (1965) who showed that it was activated by nucleoside triphosphates which did not undergo reaction. An advantage in connection with kinetic investigations of this enzyme is that it catalyzes a relatively simple reaction viz.

$$\text{nucleoside diphosphate} \xrightarrow{M^{2+}} \text{nucleoside monophosphate} + \text{inorganic phosphate}$$

As a prelude to a more detailed quantitative kinetic study of the reaction (see Chapter II), attention was directed towards obtaining from rat liver a highly purified preparation of nucleoside diphosphatase which has been used to investigate some of the general kinetic properties of the enzyme. From a study of substrate and metal requirements for the

enzyme, a selection could be made of the metal ion and nucleoside diphosphate pair which exhibited sigmoidal kinetic properties for the detailed quantitative kinetic study of the allosteric mechanism.

Experimental

Materials

The sodium salts of all nucleotides were purchased from P-L Biochemicals with the exception of dTDP and thiamine pyrophosphate (TPP) which were purchased from Calbiochem, and the phosphonate analogues of ATP which were purchased from Miles Laboratories. ATP was recrystallized twice from ethanol at 2° by the procedure of Berger (1956) and IDP, which contained about 5% IMP, was purified by a modification of the method of Morrison, O'Sullivan and Ogston (1961). A solution of IDP (1.3 g) was adsorbed onto a column (3.5 x 12 cm) of Dowex-1 (formate, 200-400 mesh) and eluted by using a linear gradient formed from 1 M formic acid and 1 M formic acid containing 2 M ammonium formate. Both mixing and reservoir vessels contained 1 l of solution. Each of the purified preparations of ATP and IDP showed the presence of only a single spot which absorbed ultraviolet light after chromatography in iso-

butyric acid-NH_3 (sp. gr. 0.88)-water (66:1:33, v/v). Stock solutions of the nucleotides were adjusted to pH 7.6 with NaOH and stored at -10°. Other nucleoside tri- and diphosphates contained small amounts of the corresponding di- and monophosphates, but were used without further purification. Concentrations of all nucleotides were checked by measurement of their absorption according to Bock et al. (1956). $MgCl_2$, $CaCl_2$ and $MnCl_2$ were reagent grade products from E. Merck, Ag, Darmstadt, which were treated with dithizone in carbon tetrachloride (Morrison and Uhr, 1966). Solutions were standardized according to the procedure outlined by Morrison et al. (1961). EDTA was a laboratory reagent from British Drug Houses. Phenyl methyl sulfonyl fluoride and dithiothreitol were obtained from the California Corporation for Biochemical Research. DEAE-Sephadex and Sephadex were supplied by Pharmacia, hydroxylapatite by Bio-Rad Laboratories and cellulose by Whatman. Horse-radish peroxidase was supplied by Sigma, and creatine kinase was prepared and stored as described by Morrison et al. (1961). All other reagents were commercial products of the highest grade available and were used without further purification. 8-^{14}C ATP-Li_4 (16.8 mC

per mmole) in 50% ethanol, was obtained from Schwarz Bioresearch, Inc.

Methods

Measurement of enzyme activity. All experiments have been carried out at pH 8.5 since at this pH, all nucleoside di- and triphosphates can be considered to exist in solution only in their fully ionized forms of NDP^{3-} and NTP^{4-}, respectively. Thus the only metal-nucleotide complexes that have to be taken into account are $MNDP^{-}$ and $MNTP^{2-}$ (O'Sullivan and Perrin, 1964). Because metal nucleotide diphosphates function as substrates for and metal nucleoside triphosphates as activators of the reaction (see RESULTS), these complexes were used as the variable reactants while the free NDP^{3-} was held constant. The concentrations of total NDP and metal chloride to give the required concentrations of $MNDP^{-}$, while maintaining free NDP^{3-} at the desired concentration, were calculated as described by Morrison et al. (1961). For this purpose it was assumed that the stability constants for all metal-nucleoside diphosphate complexes were the same as those determined for the various metal complexes of ADP (Walaas, 1958). Thus the stability constants for $MNDP^{-}$ were taken to be 4,000 M^{-1}, 25,000 M^{-1} and 2,200 M^{-1}

when M was magnesium, manganese and calcium, respectively (O'Sullivan and Perrin, 1964). As these same authors also showed that the stability constant for $MgATP^{2-}$ has a relatively high value of 73,000 M^{-1}, this complex was formed by the addition of equimolar amounts of $MgCl_2$ and ATP. Other nucleoside triphosphates were assumed to bind magnesium with the same affinity as ATP (Walaas, 1958), and thus the metal complexes were formed by the equimolar addition of nucleotide and $MgCl_2$.

Reaction mixtures contained in a total volume of either 1.0 or 2.0 ml : triethanolamine-HCl buffer, 0.1 M; EDTA, 0.01 mM and enzyme, as well as the indicated concentrations of substrate (and in some cases activators). After the addition of the components and before the addition of enzyme (0.2-0.8 µg of protein), the tubes were incubated for 3 min. at 30°. The enzyme was diluted with 0.1 M Tris-HCl buffer containing 0.01 mM EDTA and 0.1 mM dithiothreitol (pH 8.0) and added to the reaction mixtures in a volume of 1 to 4 µl. For the addition of enzyme a Hamilton micro-syringe was fitted with a Hamilton repeating dispenser which delivers one-fiftieth of the syringe volume per delivery. All experiments

were run for two time periods, between 1 and 60 min., to ensure that initial velocities were being measured, and the reaction was stopped in a manner depending on which product was to be determined. Extinction measurements were made using either a Shimadzu or Gilford 300 spectrophotometer.

A unit of activity was taken to be the amount of enzyme which releases 1 μmole of IMP or inorganic phosphate per min. from 0.5 mM $MgIDP^-$ under the above conditions.

Estimation of inorganic phosphate. The reaction was stopped by the addition of 0.1 ml of 3.5 N or 7.0 N H_2SO_4 depending on whether the volume of the reaction mixture was 1.0 or 2.0 ml. The tubes were mixed on a vortex mixer and were then plunged into ice to minimize the acid hydrolysis of nucleotide and warmed to room temperature before the addition of 0.2 ml of 1% (w/v) p-methylaminophenol sulphate in 3% (w/v) sodium sulphite to 1.0 ml of reaction mixture or equivalent amounts of the reagents to 2.0 ml of reaction mixture. This was followed by the addition of 0.5 ml of 2.5% (w/v) ammonium molybdate in 1 N H_2SO_4 to 1.0 ml of reaction mixture or 0.5 ml of 5% (w/v) ammonium molybdate in 2 M H_2SO_4 to 2.0 ml of reaction mixture. The solution of acid molybdate was added to the tubes at 12-25 secs. intervals

and the extinction measurements made at 650 mμ after 8 min. at room temperature.

Estimation of IMP. The reaction was stopped by the addition of 0.1 ml of 3.5 M HCl to 1.0 or 2.0 ml of reaction mixture and the tubes placed in ice. IMP was determined using a specific IMP dehydrogenase prepared from extracts of Aerobacter aerogenes, strain P14 and free of $NADH_2$ oxidase (Magasanik, Moyed and Gehring, 1957). Before assaying, the contents of the tubes were neutralized by the addition of 0.1 ml of 3.5 N KOH after which was added 0.27 or 0.55 ml of solution (for 1 and 2 ml reaction mixtures, respectively) containing Tris-HCl buffer (0.25 M, pH 7.6), NAD (5.1 mM), dithiothreitol (0.25 mM) and partially purified IMP dehydrogenase (0.1 mg of protein). Incubation was carried out at 30° until the reaction was complete (about 40 min.). The formation of $NADH_2$ was determined at 340 mμ in a cell of 1 or 2 cm light path. To allow for the presence of small amounts of IMP in the solutions of IDP, blanks contained all components except the nucleoside diphosphatase.

Reaction velocities were determined by measurement of inorganic phosphate production when nucleotides other than IDP were added as substrates or when

only a narrow range of relatively high substrate concentrations was used. For wider ranges of substrate concentrations which extended down to relatively low values, estimation of IMP proved to be more satisfactory. There was good agreement between the results obtained with the two methods.

Separation of nucleotides. Nucleotides were separated by chromatography on DEAE-cellulose paper by the method described by Morrison (1968).

Determination of protein. This was carried out by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Fractionation with ammonium sulphate. The weight (w) of ammonium sulphate required to give the various degrees of saturation was calculated from the formula

$$w = \frac{0.515\ V(S_2-S_1)}{1.0-0.292\ S_2}$$

where V represents the volume in millilitres and w is expressed in grams. S_1 and S_2 represent the initial and desired degrees (0-1.0) of saturation at 0° (Kunitz, 1952; Noltmann, Gubler and Kuby, 1961).

Results

Purification of nucleoside diphosphatase

The enzyme was purified by a modification of an unpublished method which was communicated by Professor O. Hayaishi. The introduction of certain modifications had the effect of increasing the yield and stability of the enzyme. The procedure is given below.

Preparation of liver acetone powder. Livers were obtained from adult white rats and dropped immediately into cold distilled water. After several minutes, they were removed, placed in redistilled acetone (approximately 10 vol. at -10°) and treated in a Waring blendor until the suspension appeared uniform (2-4 min.). The mixture was then filtered on a Buchner funnel and the pad treated again with acetone as described above. The pad was broken up and left at room temperature overnight after which it was ground in a mortar. The powder was stored at -10°. Under these conditions, the loss of nucleoside diphosphatase activity was 25% over a period of ten months. Sixty rat livers yielded 160 g of acetone powder.

Extraction of acetone powder. Unless otherwise stated, all operations were carried out at 4°, buffers contained EDTA (0.01 mM) and dithiothreitol (0.1 mM) and centrifugations were done at 13,000 g for 20 min. Dialyses were carried out against either two changes of 15 vol. or 100 vol. of buffer solution. Acetone powder (120 g) was extracted by stirring for 1 hr. with 720 ml of 0.05 M Tris-HCl buffer containing 0.25 mM phenyl methyl sulfonyl fluoride (pH 7.8). The suspension was centrifuged and the precipitate re-extracted with 240 ml of the same buffer solution as described above. The supernatant solutions were combined.

First ammonium sulphate fractionation. The extract was brought to 0.3 saturation by the addition of solid ammonium sulphate over a period of 2 hr. and after stirring for a further 15 min., the precipitate was removed by centrifugation. Solid ammonium sulphate was then added to the supernatant solution over a period of 1 hr. to bring it to 0.7 saturation. After the addition was complete, stirring was continued for an additional 15 min. before the precipitate was dissolved in 300 ml of 0.05 M acetate buffer (pH 6.0) and dialyzed for 17 hr. against the same buffer.

Fractionation on carboxymethyl (CM)-Sephadex.

CM-Sephadex (C-50) which had been equilibrated against acetate buffer (0.05 M, pH 6.0) was added to a coarse sintered glass funnel, containing filter paper (Whatman No. 531), so as to give a pad (6 x 6 cm) after the application of gentle suction. The dialyzed solution from the previous step was passed, with gentle suction, through the pad which was then washed with 2 x 100 ml lots of acetate buffer.

Second ammonium sulphate fractionation. The filtrate was fractionated with solid ammonium sulphate in a manner similar to that outlined above and the fraction precipitating between 0.4 and 0.6 saturation was collected by centrifuging. The precipitate was dissolved in 40 ml of 0.01 M Tris-HCl buffer containing 0.2 M NaCl (pH 8.0) and dialyzed for 17 hr. against the same buffer.

First chromatography on DEAE-Sephadex. A column (4.5 x 22 cm) of DEAE-Sephadex (A-50) was equilibrated against the Tris-HCl buffer referred to above. The dialyzed solution from the previous step was applied to the column and the enzyme eluted by using a linear gradient formed from 0.05 M Tris-HCl containing 0.2 M NaCl (pH 8.0) and the same buffer containing 0.35 M

NaCl. The total volume of the elution buffers was 21. Fractions (25 ml) were collected and those with enzyme of the highest specific activity (68-96 of Peak 2, Fig. I.1A) were pooled.

Second chromatography on DEAE-Sephadex. The solution of pooled fractions was diluted with Tris-HCl buffer (0.05 M, pH 8.0) so as to reduce the concentration of NaCl to approximately 0.2 M and applied to a second column (2 x 23 cm) of DEAE-Sephadex which had been prepared as described above. The same procedure as in the previous step was used for elution of the enzyme, except that each vessel contained 600 ml of the appropriate buffer solutions. Fractions (14 ml) containing enzyme of the highest specific activities (8-19 of Peaks 1 and 2 of Fig. I.1B) were pooled and dialyzed for 17 hr. against 0.005 M phosphate buffer (pH 6.8).

Chromatography on cellulose-hydroxylapatite. A column (3.5 x 4 cm) of a 1:1 (w/w) mixture of cellulose and hydroxylapatite was prepared and washed with 500 ml of the above phosphate buffer. The solution from the previous step was applied and the enzyme eluted with two successive linear gradients of phosphate buffer (pH 6.8) from 0.01-0.12 M and from

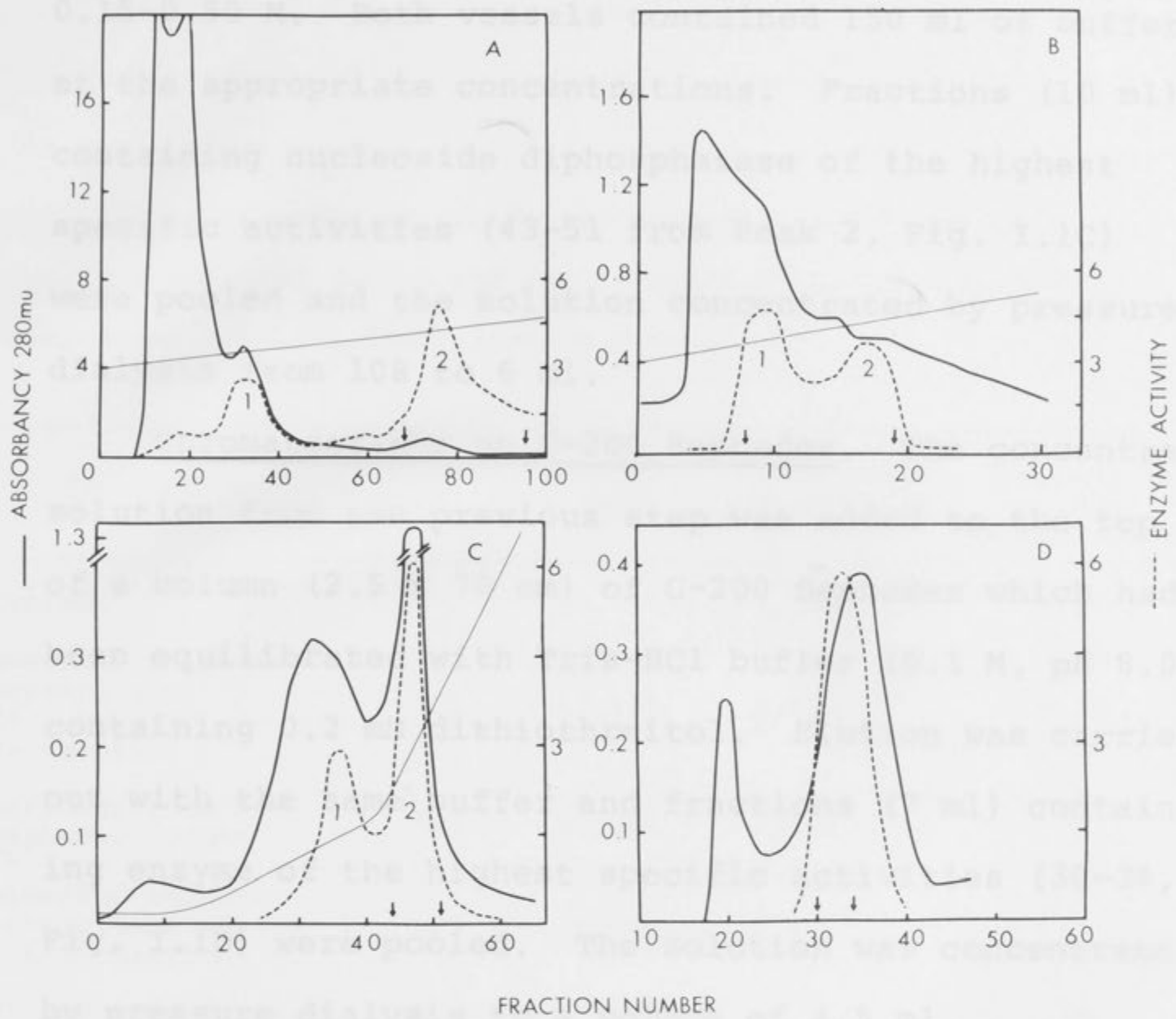


FIGURE I.1. Patterns for the elution of protein (——) and enzyme (---) from columns of : A, DEAE-Sephadex (1st); B, DEAE-Sephadex (2nd); C, hydroxyl-apatite-cellulose; D, G-200 Sephadex. The fine lines represent the rate of increase in the concentration of the eluting gradients which were formed as described in the text. The arrows indicate the enzyme fractions taken for further treatment. Enzyme activity is given in arbitrary units.

0.15-0.50 M. Both vessels contained 150 ml of buffer at the appropriate concentrations. Fractions (10 ml) containing nucleoside diphosphatase of the highest specific activities (43-51 from Peak 2, Fig. I.1C) were pooled and the solution concentrated by pressure dialysis from 108 to 6 ml.

Chromatography on G-200 Sephadex. The concentrated solution from the previous step was added to the top of a column (2.5 x 70 cm) of G-200 Sephadex which had been equilibrated with Tris-HCl buffer (0.1 M, pH 8.0) containing 0.2 mM dithiothreitol. Elution was carried out with the same buffer and fractions (7 ml) containing enzyme of the highest specific activities (30-34, Fig. I.1D) were pooled. The solution was concentrated by pressure dialysis to a volume of 4.5 ml.

A summary of the yields and specific activities of the various fractions obtained during the purification procedure is given in Table I.1.

Properties of the purified enzyme. Storage of the enzyme at concentrations of 1 to 3 mg per ml at 5° in the presence of Tris-HCl buffer (0.1 M, pH 8.0) containing 0.2 mM dithiothreitol and 0.01 mM EDTA resulted in a variable loss of activity of up to 20% per week. When such solutions were frozen by immersion

TABLE I.1. : Summary of yields and specific activities of fractions obtained during the purification of nucleoside diphosphatase from rat liver acetone powder[a].

Fraction	Volume (ml)	Protein (mg)	Total Units (μmoles/min)	Specific Activity (mμmoles/min/μg)
Extract	985	43,750	3,060	0.07
Ammonium sulphate precipitate (0.3 to 0.7 saturation)	375	33,750	2,470	0.07
Effluent from CM-Sephadex column	440	20,240	2,630	0.13
Ammonium sulphate precipitate (0.4 to 0.6 saturation)	104	9,980	1,530	0.15
Eluate from first DEAE-Sephadex column	750	495	1,070	2.16
Eluate from second DEAE-Sephadex column	154	103	1,010	9.8
Eluate from hydroxylapatite column	5.8	64	460	7.2
Eluate from G-200 Sephadex column	4.5	10.3	610	59.2

[a]Weight of rat liver acetone powder was 120 g. Details are given in the text.

in dry ice-ethanol and stored at -10°, there was no change in specific activity for periods up to eight months. However, after 3-4 months of storage, the enzyme exhibited normal Michaelis-Menten kinetics in contrast to the non-linear kinetics shown by preparations which had been stored for shorter periods (cf. Figs. I.6 and I.13). Repeated freezing and thawing had no effect on the activity of the enzyme.

Polyacrylamide gel electrophoresis of the purified enzyme showed the presence of 5-6 bands (Fig. I.2). It is possible that more than one band possesses enzyme activity, especially since the elution patterns from columns of DEAE-Sephadex and hydroxylapatite (Fig. I.1) suggest that the enzyme may undergo association-dissociation reactions. However, a comparison of the kinetic properties of nucleoside diphosphatase, as present in the two fractions obtained by elution of the second DEAE-Sephadex column (Fig. I.1B) has shown that no differences could be detected. All subsequent kinetic studies have been made with the most highly purified preparation of the enzyme (Table I.1). As the specific activity of this preparation is similar to that of an apparently pure preparation of nucleoside diphosphatase from bovine liver microsomes (Yamazaki

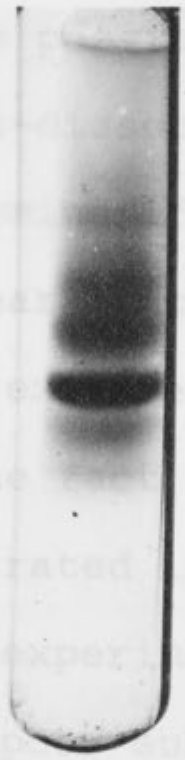


FIGURE I.2. Separation of the protein components of the purified nucleoside diphosphatase (50 µg) on polyacrylamide gel. The unpublished procedure of Orr and Blakley was used.

and Hayaishi, 1968), it may be approaching a pure state. A further indication of the state of purity is given by the fact that in some preparations, the leading edge of the enzyme peak as eluted from G-200 Sephadex (Fig. I.1D) had a constant specific activity.

In view of the possibility that the enzyme could undergo association-dissociation reactions, it was of importance to determine if the initial velocity of the reaction was a linear function of enzyme concentration over a range which exceeded that used in the kinetic investigations. The fact that such a relationship does hold is illustrated in Fig. I.3. With the exception of those experiments carried out with what can be regarded as poor substrates, the concentrations of enzyme fell within the range covered by the arrows in Fig. I.3.

Estimation of the molecular weight of the enzyme. Experiments were carried out in order to estimate the molecular weight of nucleoside diphosphatase and to detect any polymerization reaction that could result when the allosteric activator was added or when upon long periods of storage of the enzyme, its kinetic properties change. In order to carry out these experiments, the gel-filtration technique described

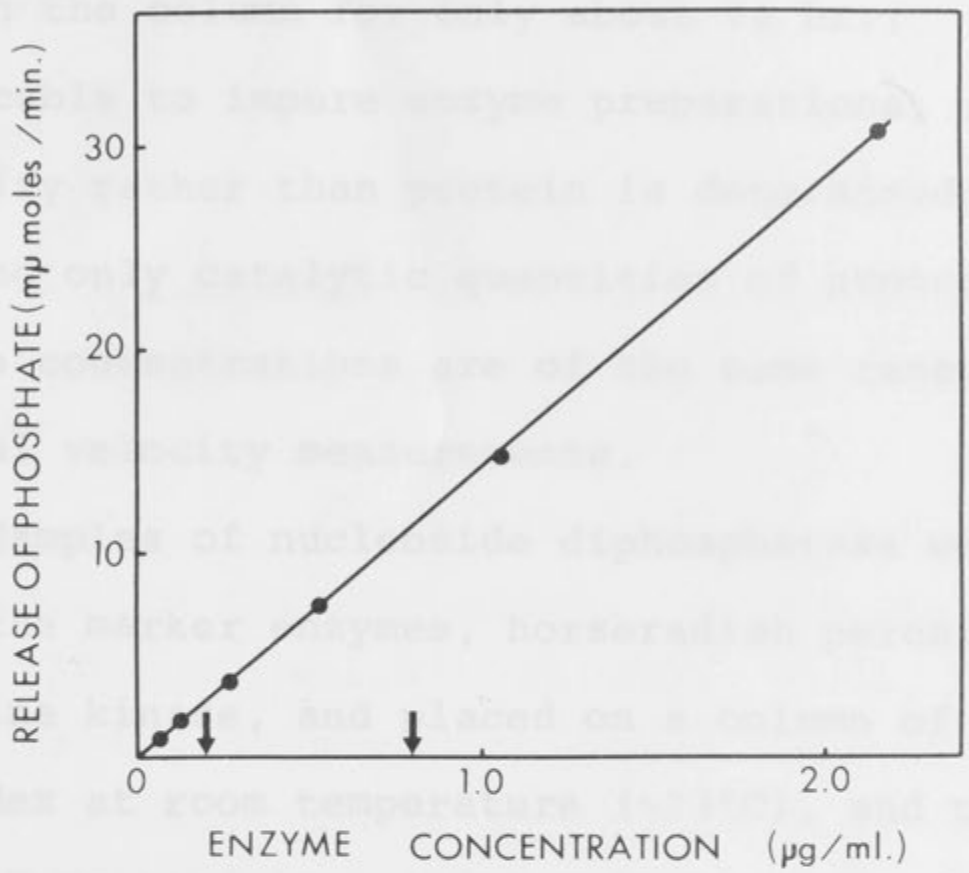


FIGURE I.3. Initial velocity of the reaction as a function of the enzyme concentration at a $MgIDP^-$ concentration of 2.5 mM. The arrows indicate the range of enzyme concentrations used in most experiments.

by Andrews (1964) was used. Although this method of molecular weight determination is relatively inaccurate, it has the advantages of (a) being rapid, as the enzymes are on the column for only about 1½ hr.; (b) being applicable to impure enzyme preparations, as enzymic activity rather than protein is determined; and (c) requiring only catalytic quantities of protein, so that enzyme concentrations are of the same ranges used in initial velocity measurements.

Samples of nucleoside diphosphatase were mixed with the marker enzymes, horseradish peroxidase and creatine kinase, and placed on a column of G-200 Sephadex at room temperature (∿23°C), and pH 8.5, the same pH as used in initial velocity measurements. The enzymes were eluted and fractions of constant volume were collected for determination of enzymatic activity. The fractions in which the marker enzymes were eluted were plotted as a function of log molecular weight (Andrews, 1964), and the molecular weight of nucleoside diphosphatase estimated from this plot. A typical result of this type of experiment is shown in Figure I.4.

Two molecular weight determinations gave values of 81,000 and 108,000 for nucleoside diphosphatase.

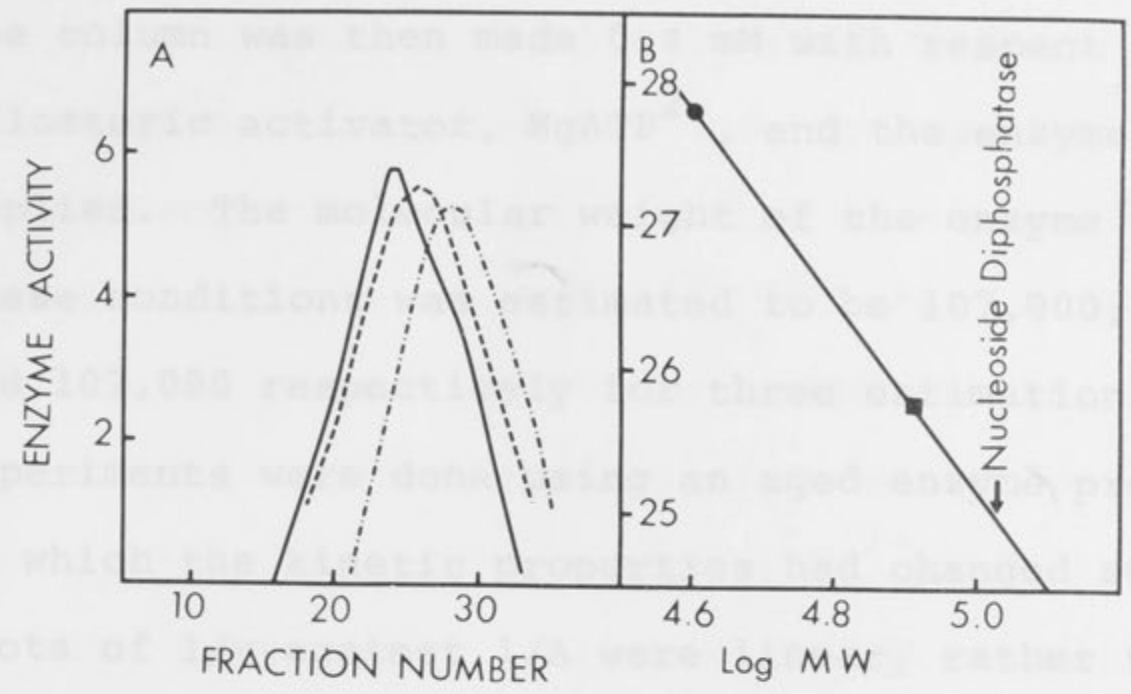


FIGURE I.4. Estimation of the molecular weight of nucleoside diphosphatase. A mixture of creatine kinase (50 µg; mol.wt. 81,000; Noda, Kuby and Lardy, 1954), horseradish peroxidase (5 µg; mol.wt. 40,000; Maehly, 1955) and nucleoside diphosphatase (20 µg) in a volume of 0.15 ml Tris-HCl buffer, 0.1 M, pH 8.5, containing 10^{-5} M EDTA, 10^{-4} M dithiothreitol, and 5 mg/ml sucrose was layered on a column (1.2 x 9.5 cm) of G-200 Sephadex and eluted with the same buffer without sucrose. In some experiments 0.4 mM $MgATP^{2-}$ was added to the buffer. Fractions of 10 drops (0.2880 g ± 0.0003 g) were collected. (A) Elution patterns of nucleoside diphosphatase (———), creatine kinase (----) and horseradish peroxidase (-.-.-.). Enzyme activities are expressed in arbitrary units. (B) A plot of fraction number against log molecular weight. Elution fraction of horseradish peroxidase (●) and creatine kinase (■).

The column was then made 0.4 mM with respect to the allosteric activator, $MgATP^{2-}$, and the enzyme solution applied. The molecular weight of the enzyme under these conditions was estimated to be 107,000; 116,000 and 107,000 respectively for three estimations. Similar experiments were done using an aged enzyme preparation in which the kinetic properties had changed such that plots of 1/v against 1/A were linear, rather than curvilinear, as are plots obtained with enzyme that has not been aged (cf. Figures I.6B and I.13). The molecular weight of this enzyme with no allosteric activator present, was estimated to be 85,000 and 120,000 respectively for two determinations. Because the elution peak of nucleoside diphosphatase is assymetric under all the conditions described above, the molecular weight values may only approximate the true molecular weight. Even so, large changes in molecular weight which would occur upon polymerization or dissociation of the enzyme were not observed. Despite the assymetric elution patterns, these molecular weight determinations are in agreement with the results of Yamazaki and Hayaishi (1968) whose ultracentrifuge data gave a molecular weight of 100,000 for nucleoside diphosphatase from bovine liver microsomes.

From the above results it would appear that nucleoside diphosphatase does not undergo a polymerization or a dissociation reaction in the presence of the allosteric activator, $MgATP^{2-}$, and that changes in the kinetic properties of the enzyme due to ageing are not related to an alteration of molecular weight.

Activation of the enzyme by Mg^{2+}. In the absence of added metal ions, there was a slow enzymatic reaction when unpurified samples of IDP were used as substrate. However, it would appear that this activity was due to the presence, in commercial preparations of IDP, of one or more bivalent metal ions which could activate the enzyme. Thus there was no hydrolysis of purified IDP unless Mg^{2+}, Ca^{2+} or Mn^{2+} was present in the reaction mixture.

To gain preliminary information about the function of metal ions, the initial velocity of the reaction was studied as a function of the concentrations of total magnesium and total IDP. The results (Fig. I.5A) indicate that the concentrations of $MgCl_2$ required to obtain an apparent maximum velocity increase as the concentration of total IDP is increased and that each apparent maximum velocity is attained under conditions where the ratio of magnesium : total IDP is greater

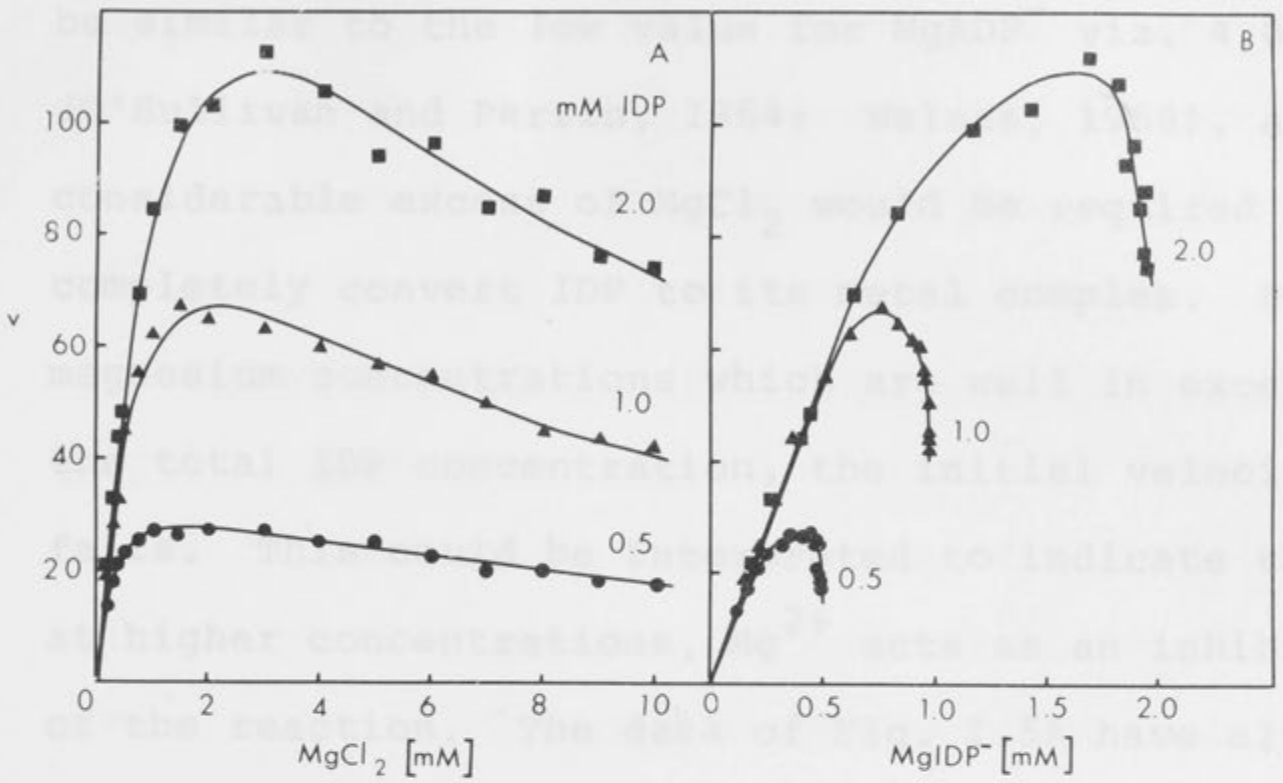


FIGURE I.5. (A) Effect of the total magnesium concentration on the initial velocity of the reaction at different fixed concentrations of IDP. (B) The relationship between enzyme activity and the concentration of $MgIDP^-$ under conditions where the concentrations of both free Mg^{2+} and free IDP^{3-} varies. The concentrations of total IDP were: 0.5 mM (●), 1.0 mM (▲) and 2.0 mM (■). Velocity is expressed as mμmoles of IMP per min per μg of protein.

than one. These results are generally in accord with those expected if $MgIDP^-$ were acting as the substrate. Because the stability constant for $MgIDP^-$ is likely to be similar to the low value for $MgADP^-$ viz. 4,000 M^{-1} (O'Sullivan and Perrin, 1964: Walaas, 1958), a considerable excess of $MgCl_2$ would be required to completely convert IDP to its metal complex. But at magnesium concentrations which are well in excess of the total IDP concentration, the initial velocity falls. This could be interpreted to indicate that, at higher concentrations, Mg^{2+} acts as an inhibitor of the reaction. The data of Fig. I.5A have also been plotted to demonstrate the reaction velocity as a function of the $MgIDP^-$ concentration (Fig. I.5B). The results clearly illustrate the inhibition that occurs under conditions where the concentration of free Mg^{2+} is high and sufficient to convert virtually all the IDP to $MgIDP^-$. If Mg^{2+} had no effect on the reaction velocity, then for this type of plot only a single curve would be obtained, irrespective of the total IDP concentration. Although it is not readily apparent from Fig. I.5B, it appeared that at similar low concentrations of $MgIDP^-$, when IDP^{3-} was present in excess, the velocity increased as a function of the

free IDP^{3-} concentration. This was the first indication that IDP^{3-} could function as an activator of the nucleoside diphosphatase reaction and the result was confirmed later (cf. Fig. I.7). In subsequent experiments, $MgIDP^{-}$ was used as the variable substrate and further investigations were made of the effects of free IDP^{3-} and free Mg^{2+} on the initial velocity of the reaction.

Effects of $MgIDP^{-}$ concentration on the initial velocity of the reaction. When free IDP^{3-} was maintained constant at a concentration of 0.1 mM, a plot of initial velocity against the concentration of $MgIDP^{-}$ gave a curve which might be considered as being sigmoidal (Fig. I.6A), but it is not possible to reach any definite conclusion about its shape. However, when the same data are plotted in double reciprocal form, it becomes clear that the kinetics of the nucleoside diphosphatase reaction are not of the Michaelis-Menten type since the plot is not linear (Fig. I.6B). Indeed, the curve has the shape of a non-rectangular hyperbola. Further, it will be noted that extrapolation of the linear portion of the curve, which appears to be an asymptote, gives a negative intercept on the ordinate. A comparison of the two types of plot shown in Fig. I.6 indicates that the plotting of data in double

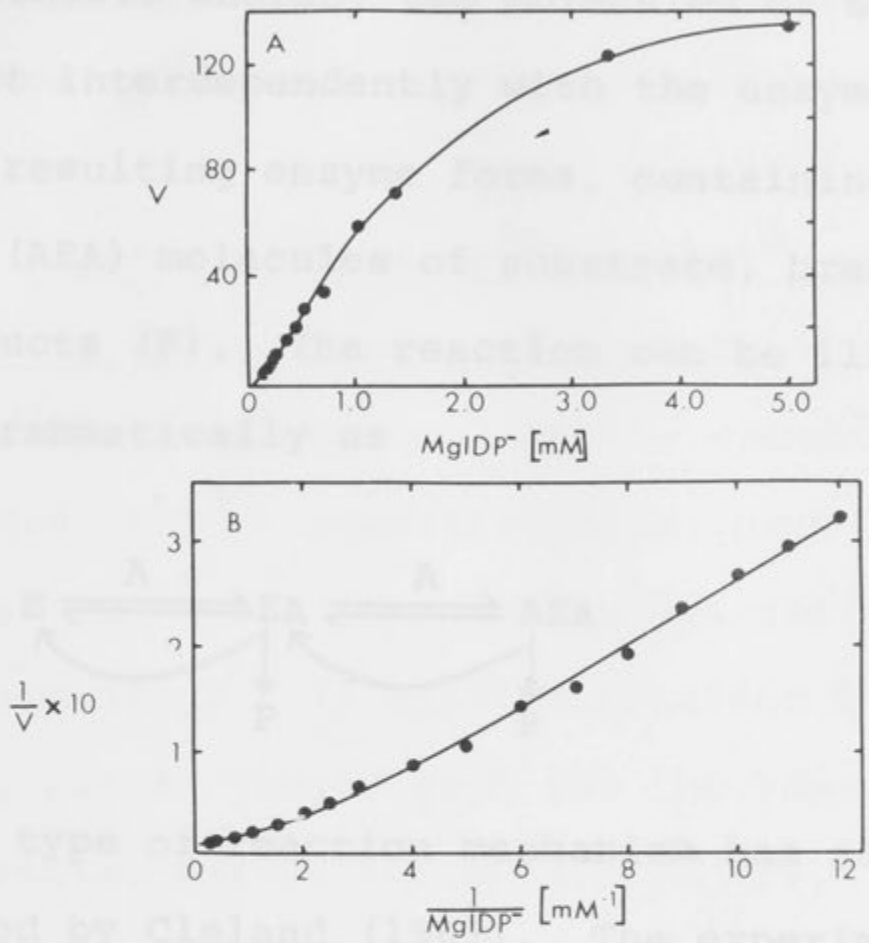
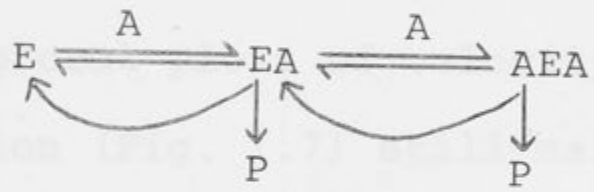


FIGURE I.6. Effect of $MgIDP^-$ concentration on the initial velocity of the reaction. The data are plotted as velocity against substrate concentration (A) and in double reciprocal form (B). Velocity is expressed as mμmoles of IMP per min per μg of protein.

reciprocal form is a far more satisfactory method of revealing non-linear reaction kinetics.

The above results are consistent with the hypothesis whereby two molecules of substrate (A) react interdependently with the enzyme (E) and both the resulting enzyme forms, containing one (EA) and two (AEA) molecules of substrate, break down to yield products (P). The reaction can be illustrated diagrammatically as



This type of reaction mechanism has also been discussed by Cleland (1967). The experimental data are not in agreement with the breakdown of only AEA for in this case, a parabolic double reciprocal plot would be obtained. The curve shown in Fig. I.6B differs from the parabola drawn by Yamazaki and Hayaishi (1965) using data obtained by varying the concentration of total IDP in the presence of a fixed concentration of $MgCl_2$. It should be pointed out that these authors could also have drawn through their experimental points at lower substrate concentrations,

a straight line which, on extrapolation, would have given a negative intercept on the ordinate. But because of the differences in the approach used in the present work and that of Yamazaki and Hayaishi (1965), no quantitative comparison can be made of the two sets of results.

Effects of free Mg^{2+} and IDP^{3-} on the initial velocity of the reaction. If the concentration of free Mg^{2+} were held constant at a concentration of 20 mM and the free IDP^{3-} concentration allowed to vary, double reciprocal plots of velocity against $MgIDP^{-}$ concentration (Fig. I.7) still maintained their non-linear form, but as judged from the increased slope of the asymptote, marked inhibition occurs. Although no conclusion could be drawn from these data about the type of inhibition, it was subsequently shown to be of the non-competitive type (see Chapter II). Because of the non-competitive inhibition of the reaction by free Mg^{2+}, it is unlikely that the intersection point on the ordinate of Fig. I.6B represents the reciprocal of the true maximum velocity. Under conditions where the concentration of $MgIDP^{-}$ is increased while the concentration of free IDP^{3-} is held constant, there will be a simultaneous increase in the concentration of free

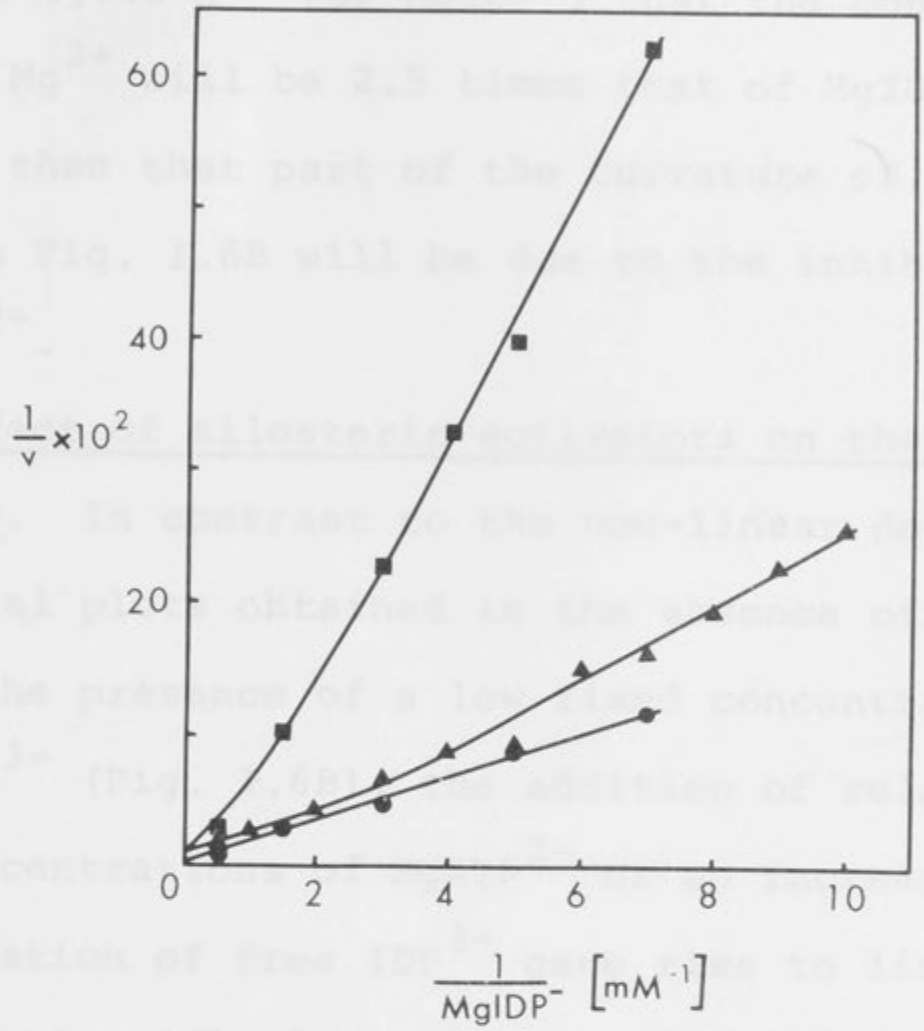


FIGURE I.7. Inhibition of the reaction by free Mg^{2+} at a concentration of 20 mM (■) and activation by free IDP^{3-} at a concentration of 1.0 mM (●). For control experiments (▲), the concentration of IDP^{3-} was held constant at 0.1 mM. Velocity is expressed as mμmoles per min per μg of protein.

Mg^{2+} to levels which cause significant inhibition. Indeed, it may be calculated, using a stability constant of 4,000 M^{-1} for $MgIDP^-$, that the concentration of free Mg^{2+} will be 2.5 times that of $MgIDP^-$. It follows then that part of the curvature of the plot shown in Fig. I.6B will be due to the inhibition by free Mg^{2+}.

Effect of allosteric activators on the reaction kinetics. In contrast to the non-linear double reciprocal plots obtained in the absence of activators and in the presence of a low fixed concentration of free IDP^{3-} (Fig. I.6B), the addition of relatively high concentrations of $MgATP^{2-}$ or an increase in the concentration of free IDP^{3-} gave rise to linear double reciprocal plots (Figs. I.7 and I.8). Thus it may be concluded that under these conditions only one molecule of substrate reacts with the enzyme[1]. In both cases it will be noted that the degree of activation decreases as the concentration of $MgIDP^-$ increases. Further, it is clear that the maximum velocity of the

---

[1]When referred to in this sense, the enzyme means an independent catalytic centre, of which more than one may exist on any enzyme molecule.

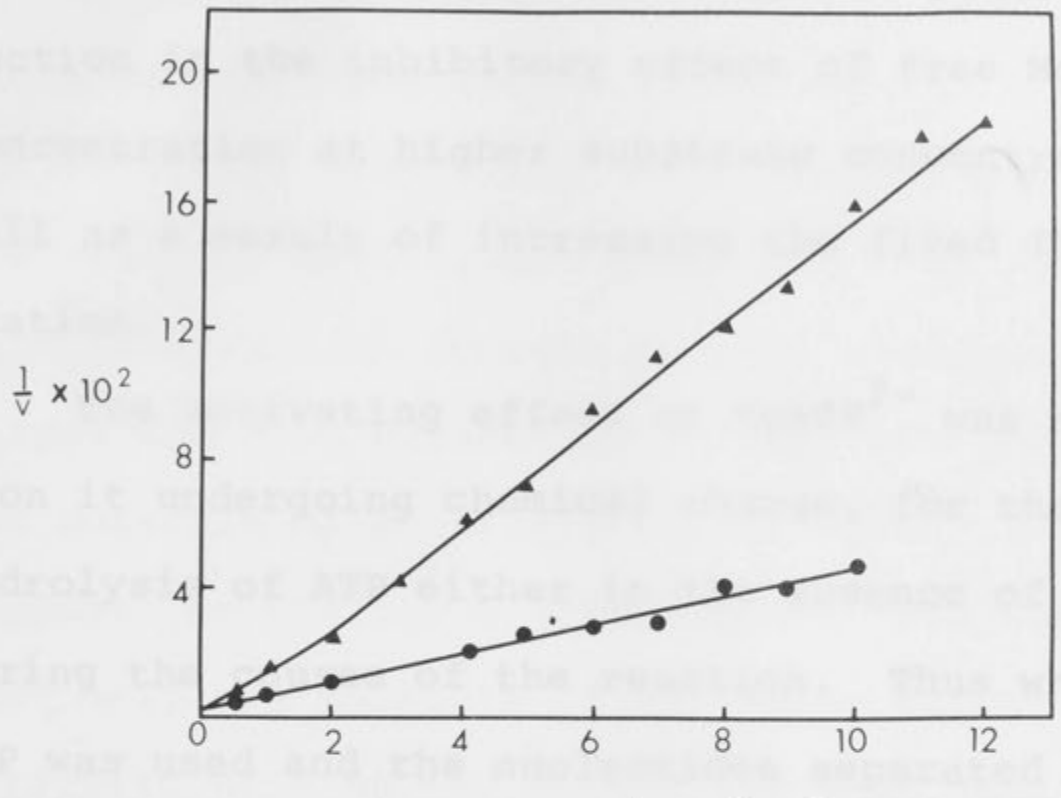
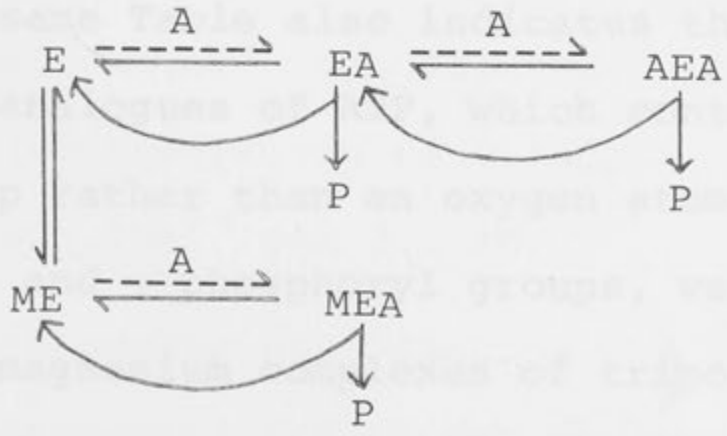


FIGURE I.8. Activation of the reaction by $MgATP^{2-}$. The concentrations of $MgATP^{2-}$ were : ▲, none; ●, 0.6 mM. IDP^{3-} was held constant at a fixed concentration of 0.1 mM. Velocity is expressed as mμmoles of IMP per min per μg of protein.

reaction is greater in the presence of 1.0 mM free IDP^{3-} than in the presence of 0.1 mM free IDP^{3-} (Fig. I.7). This finding can be explained as being due to the reduction in the inhibitory effect of free Mg^{2+} whose concentration at higher substrate concentrations must fall as a result of increasing the fixed IDP^{3-} concentration.

The activating effect of $MgATP^{2-}$ was not dependent upon it undergoing chemical change, for there was no hydrolysis of ATP either in the absence of substrate or during the course of the reaction. Thus when labelled ATP was used and the nucleotides separated by chromatography on DEAE-cellulose paper, the number of counts per min. in the ATP spot was equivalent to the number added.

The results obtained in the presence and absence of activators are in general agreement with the following scheme



in which it is supposed that only one molecule of the activator (M) combines at one of the catalytic sites on the enzyme at which substrate can react and that because of the structural similarities of the activator and substrate molecules, their combination at this site are mutually exclusive. Thus when M is present at sufficiently high concentrations, essentially all the enzyme would be present in the ME form with which only one molecule of substrate would react. Additional support for the above scheme comes from the findings that only one molecule of activator reacts with each catalytic unit of the enzyme and that high fixed concentrations of activator do not cause inhibition of the reaction (Yamazaki and Hayaishi, 1965; Chapter II).

The increase in reaction velocity, at a low substrate concentration, as a result of the addition of a fixed concentration of various magnesium-nucleoside triphosphate complexes is shown in Table I.2. The same Table also indicates that magnesium-phosphonate analogues of ATP, which contained a methylene group rather than an oxygen atom between the α and β or β and γ phosphoryl groups, were inactive as were the magnesium complexes of tripolyphosphate and trimetaphosphate. Magnesium pyrophosphate, on the other

TABLE I.2. : Effect of magnesium complexes of nucleotides and polyphosphates on the rate of hydrolysis of $MgIDP^-$ [a].

Additions	Initial velocity (mμmoles/min/μg)
None	18.5
$MgATP^{2-}$	46.8
$MgITP^{2-}$	54.7
$MgGTP^{2-}$	52.3
$MgUTP^{2-}$	37.7
$MgCTP^{2-}$	28.8
β-γ Phosphonate analogue of $MgATP^{2-}$ [b]	17.8
α-β Phosphonate analogue of $MgATP^{2-}$ [c]	20.1
Mg-pyrophosphate	13.9
Mg-tripolyphosphate	19.5
Mg-trimetaphosphate	19.0

[a] Both $MgIDP^-$ and the test compounds were present at a concentration of 0.143 mM. The concentration of free IDP^{3-} was 0.1 mM.

[b,c] The oxygen atom linking the β and γ or α and β phosphoryl groups was replaced by a methylene group.

hand, caused inhibition of the reaction.

Substrate specificity of the enzyme. To determine the relative rates of hydrolysis of the magnesium complexes of various nucleoside diphosphate complexes, their reaction velocities were measured at two different concentrations both in the presence and absence of the allosteric activator, $MgATP^{2-}$. From the results illustrated in Table I.3, it may be concluded that the nucleotides fall into three classes according to their rates of hydrolysis. The magnesium complexes of IDP, UDP and GDP can be considered as good substrates since they undergo reaction at much greater rates than the magnesium complexes of CDP and ADP which hence can be regarded as poor substrates. The evidence suggests that $MgdTDP^-$ is not a substrate for nucleoside diphosphatase, although the enzyme is capable of hydrolyzing thiamine pyrophosphate.

At a substrate concentration of 0.5 mM, the good substrates, $MgIDP^-$, $MgUDP^-$ and $MgGDP^-$, underwent reaction at comparable rates, but at the lower substrate concentration of 0.143 mM, the relative rates of hydrolysis decreased in the order : $MgUDP^-$ > $MgGDP^-$ > $MgIDP^-$. The rates of hydrolysis of all substrates at both concentrations were increased

TABLE I.3. : Effect of $MgATP^{2-}$ on the rate of hydrolysis of various magnesium nucleoside diphosphate complexes and magnesium thiamine pyrophosphate[a].

Substrate	Initial velocity (mμmoles/min/μg) Substrate concentration 0.5 mM		0.143 mM	
	$-MgATP^{2-}$	$+MgATP^{2-}$	$-MgATP^{2-}$	$+MgATP^{2-}$
$MgIDP^-$	65.5	108.0	18.1	40.2
$MgUDP^-$	64.1	92.0	25.8	39.0
$MgGDP^-$	63.0	98.5	21.4	41.2
$MgCDP^-$	3.3	20.4	0.88	4.2
$MgADP^-$	0.23	0.60	0.04	0.15
$MgdTDP^-$	<0.01	<0.03	<0.002	<0.002
Mg thiamine pyrophosphate	2.0	12.9	0.29	3.06

[a]Substrates and $MgATP^{2-}$ were present at equimolar concentrations.

considerably on the addition of an equimolar concentration of $MgATP^{2-}$. The relative increase in velocity ranged from 1.5 to 10.6 with $MgUDP^{-}$ and magnesium thiamine pyrophosphate, respectively, as substrates. In so far as the results with IDP, UDP, GDP and thiamine pyrophosphate are concerned, they are similar to those reported by Yamazaki and Hayaishi (1965).

A more detailed study of the kinetics of the hydrolysis of the good substrates showed that, with free NDP^{3-} at a fixed concentration of 0.1 mM, $MgGDP^{-}$ is similar to $MgIDP^{-}$ in giving non-linear double reciprocal plots while $MgUDP^{-}$ differs in that it gives a linear plot (Fig. I.9). The latter result is not inconsistent with the idea that two molecules of each substrate react with the enzyme. If the binding of the first molecule of $MgUDP^{-}$ were considerably stronger than that of the second, then it is possible that over the substrate concentration range used, only the reaction of the second molecule would be detected. Another feasible explanation is that UDP^{3-} is a much better activator than either IDP^{3-} or GDP^{3-}. It would appear from Fig. I.9 that the same maximum velocity is obtained with these three substrates. Provided that the inhibitory effect of free Mg^{2+} does not vary with the substrate,

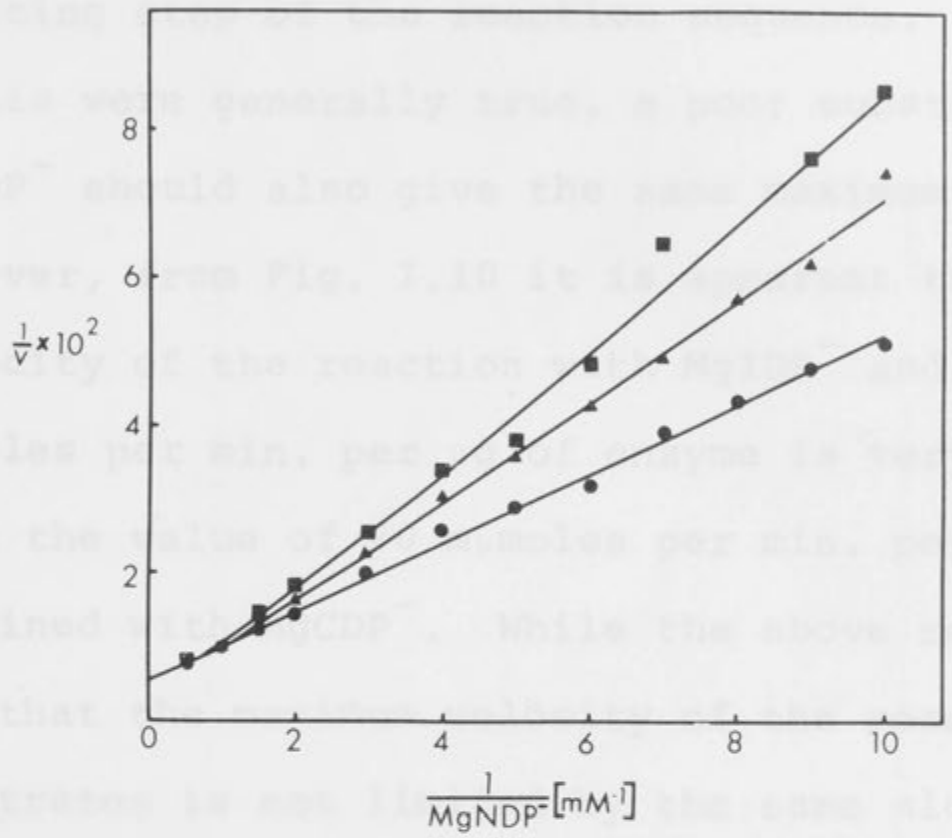


FIGURE I.9. Effect of the concentration of various magnesium nucleoside diphosphate complexes ($MgNDP^-$) on the initial velocity of the reaction. ●, $MgUDP^-$; ▲, $MgGDP^-$; ■, $MgIDP^-$. The free concentration of each nucleoside diphosphate was maintained constant at 0.1 mM. Velocity is expressed as mμmoles of inorganic phosphate per min per μg of protein.

this result suggests that a common intermediate reaction involving the release of a phosphoryl group from an enzyme-phosphate complex could be the rate-limiting step of the reaction sequence. If this hypothesis were generally true, a poor substrate such as $MgCDP^-$ should also give the same maximum velocity. However, from Fig. I.10 it is apparent that the maximum velocity of the reaction with $MgIDP^-$ and $MgUDP^-$ of 170 mμmoles per min. per μg of enzyme is very much higher than the value of 20 mμmoles per min. per μg of enzyme obtained with $MgCDP^-$. While the above results indicate that the maximum velocity of the reactions with all substrates is not limited by the same slow step, they do not preclude the conclusion that the rate-limiting step with good substrates is the hydrolysis of an enzyme-phosphate complex. It is possible that certain metal-nucleotide complexes are poor substrates because the reaction velocity is limited by the even slower rate of release of the nucleoside monophosphate moiety. The apparent linearity of the plots with $MgIDP^-$ and $MgCDP^-$ as the variable substrates, as shown in Fig. I.10, is due to the use of a higher range of substrate concentrations over which double reciprocal plots approximate to a straight line.

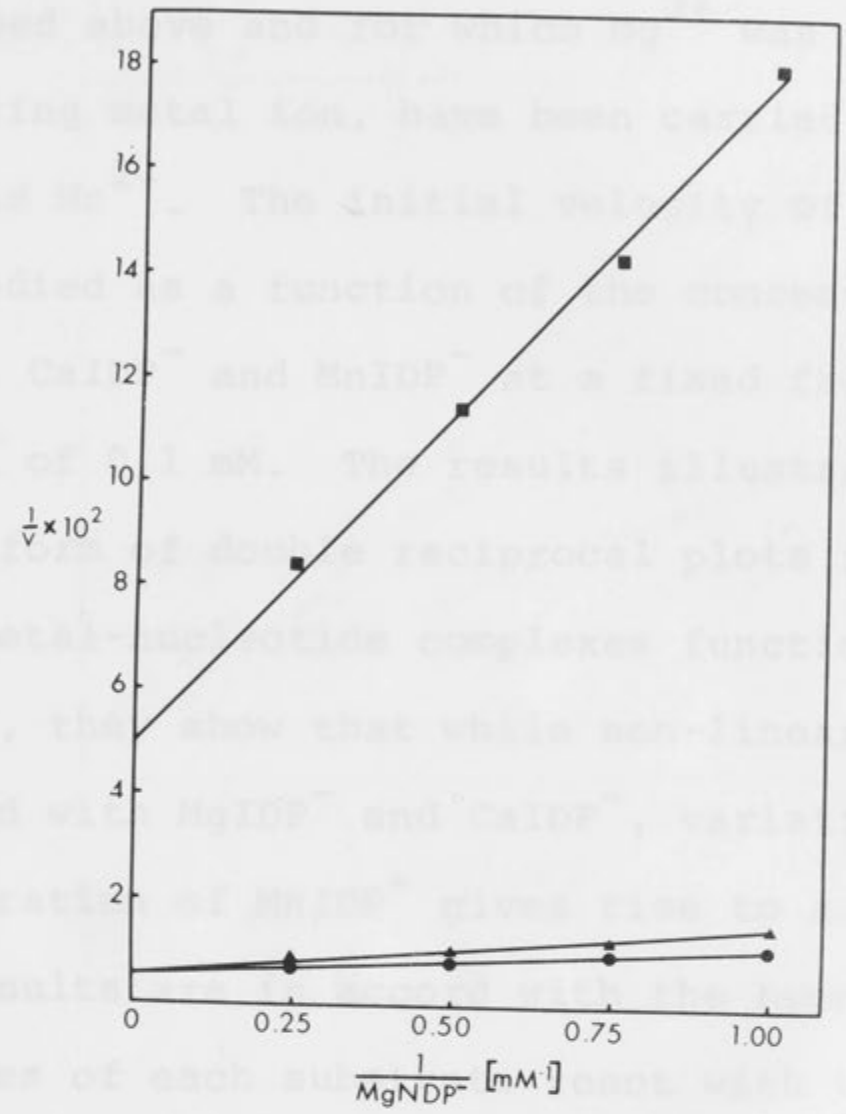


FIGURE I.10. Determination of the maximum velocity of the reaction with $MgUDP^-$ (●), $MgIDP^-$ (▲) and $MgCDP^-$ (■) as substrates. The free nucleotide concentration was 0.1 mM. Velocity is expressed as mμmoles of inorganic phosphate per min per μg of protein.

Initial velocity studies with Mg^{2+}, Ca^{2+} and Mn^{2+} as activating metal ions. Experiments similar to those described above and for which Mg^{2+} was used as the activating metal ion, have been carried out with both Ca^{2+} and Mn^{2+}. The initial velocity of the reaction was studied as a function of the concentrations of $MgIDP^-$, $CaIDP^-$ and $MnIDP^-$ at a fixed free IDP^{3-} concentration of 0.1 mM. The results illustrated in Fig. I.11 in the form of double reciprocal plots indicate that all three metal-nucleotide complexes function as substrates. Further, they show that while non-linear plots are obtained with $MgIDP^-$ and $CaIDP^-$, variation of the concentration of $MnIDP^-$ gives rise to a linear plot. Such results are in accord with the hypothesis that two molecules of each substrate react with the enzyme, although it is necessary to postulate that the first molecule of $MnIDP^-$ combines more strongly than the second. This explanation is similar to that given above in connection with the hydrolysis of $MgUDP^-$.

It is difficult to reach any firm conclusion about the relative magnitudes of the maximum velocities with each of the three metal-IDP substrates. They certainly do not appear to differ greatly, but any comparison must relate to the true maximum velocities.

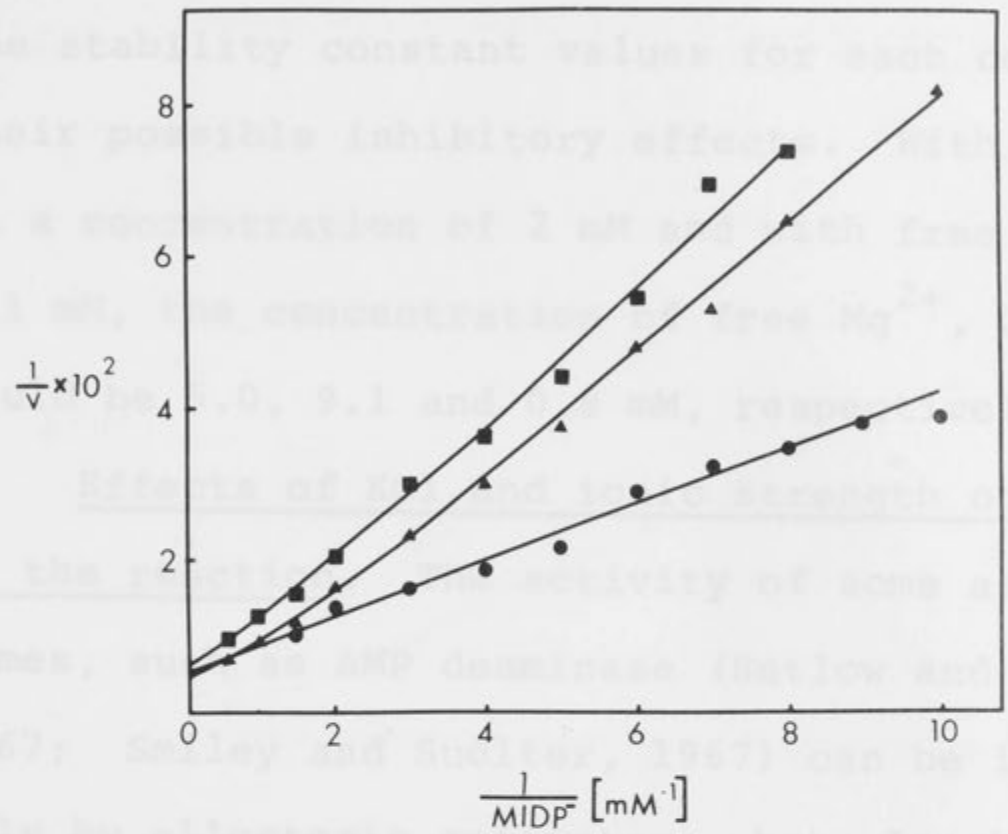


FIGURE I.11. Effect of the concentration of various metal-IDP complexes ($MIDP^-$) on the initial velocity of the reaction. ●, $MnIDP^-$; ▲, $MgIDP^-$; ■, $CaIDP^-$. The concentration of IDP^{3-} was held constant at 0.1 mM. Velocity is expressed as mμmoles of IMP per min per μg of protein.

To do this, it is essential to take into account the variation in the concentrations of each free metal ion species, which will occur because of the differences in the stability constant values for each complex, and their possible inhibitory effects. With each substrate at a concentration of 2 mM and with free IDP^{3-} fixed at 0.1 mM, the concentration of free Mg^{2+}, Ca^{2+} and Mn^{2+} would be 5.0, 9.1 and 0.8 mM, respectively.

Effects of KCl and ionic strength on the kinetics of the reaction. The activity of some allosteric enzymes, such as AMP deaminase (Setlow and Lowenstein, 1967; Smiley and Suelter, 1967) can be increased not only by allosteric activators, but also by higher concentrations of monovalent cations and/or an increase in ionic strength. Thus it was of interest to determine if such factors could cause activation of nucleoside diphosphatase. From the results of Fig. I.12, it is apparent that, at a concentration of 0.1 M, KCl acts as an inhibitor, rather than an activator, of the enzyme and does not cause any change in the general shape of the double reciprocal plot. The inhibition by KCl is not due simply to an increase in ionic strength for a two-fold increase in the concentration of triethanolamine-HCl buffer from 0.1 to 0.2 M is

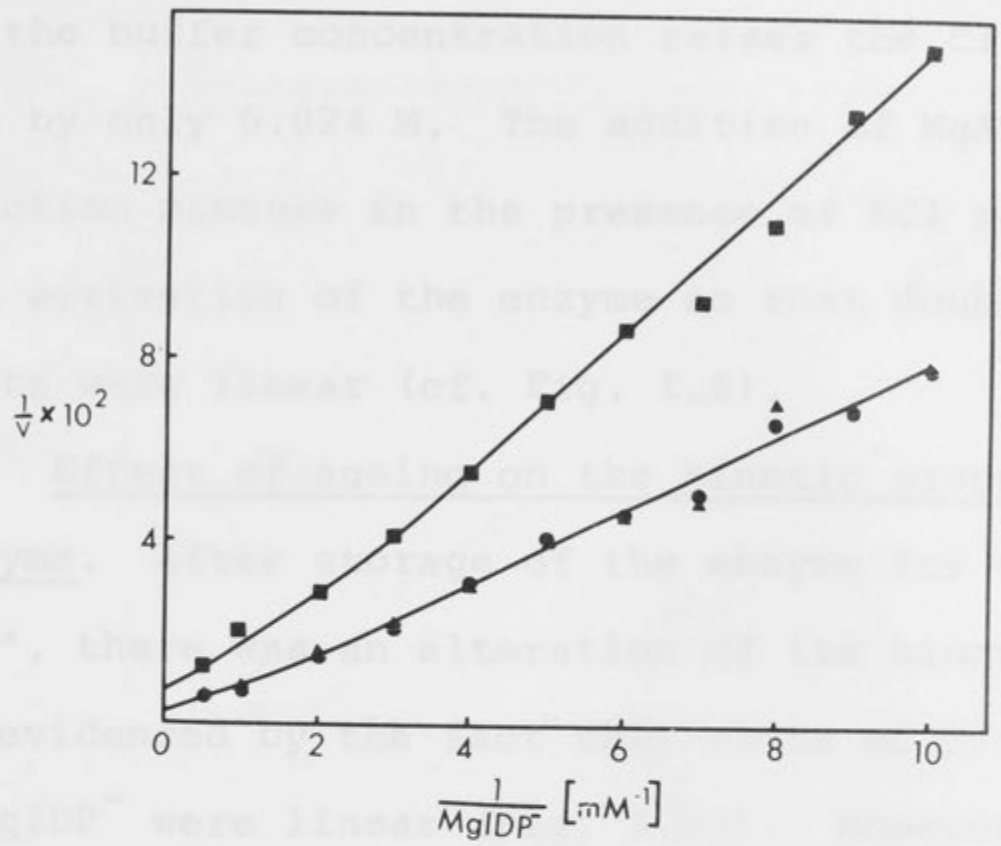


FIGURE I.12. Effect of ionic strength and KCl on the initial velocity of the reaction. The concentrations of triethanolamine-HCl buffer (pH 8.5) were 0.1 M (●) and 0.2 M (▲). KCl (■) was added at a concentration of 0.1 M to the reaction mixture containing 0.1 M triethanolamine-HCl buffer. Velocity is expressed as mμmoles of IMP per min per μg of protein.

without effect. The latter result makes it appear likely that K^+ and not Cl^- is the effective agent, but it should be borne in mind that an increase of 0.1 M in the buffer concentration raises the Cl^- concentration by only 0.024 M. The addition of $MgATP^{2-}$ to the reaction mixture in the presence of KCl resulted in the activation of the enzyme so that double reciprocal plots were linear (cf. Fig. I.8).

Effect of ageing on the kinetic properties of the enzyme. After storage of the enzyme for four months at -10°, there was an alteration of its kinetic properties as evidenced by the fact that plots of 1/v against $1/MgIDP^-$ were linear (Fig. I.13). However, the enzyme was still capable of being activated by $MgATP^{2-}$ and there was no change in its specific activity. This change in reaction mechanism, is not associated with a gross alteration of the physical size of the enzyme, as can be seen by the molecular weight determination results reported earlier.

Relative stability of the catalytic and allosteric properties. In view of the fact that the ability of nucleoside diphosphatase to be activated by $MgATP^{2-}$ was unaffected by high ionic strengths (Fig. I.12) and by prolonged ageing (Fig. I.13), further studies were

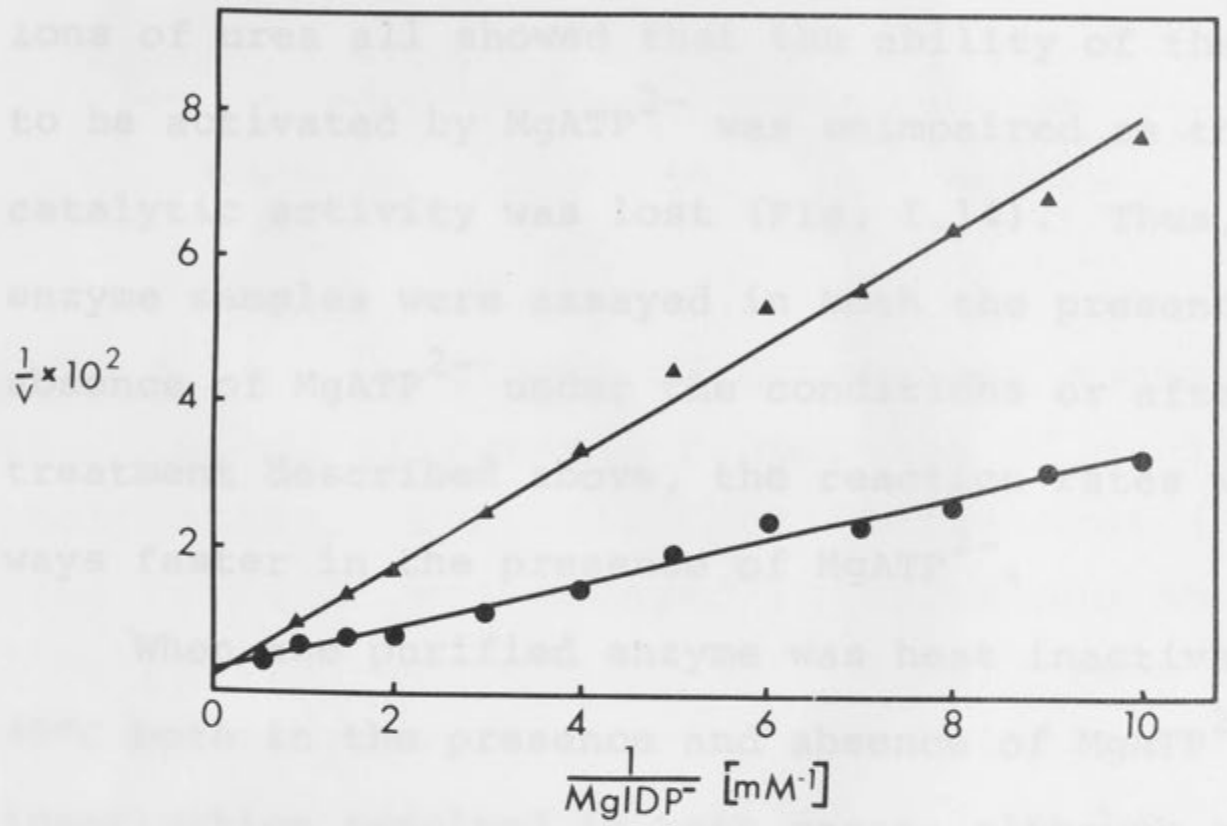


FIGURE I.13. Effect of the concentration of $MgIDP^-$ on the initial velocity of the reaction catalysed by an aged enzyme preparation in the absence (▲) and presence (●) of $MgATP^{2-}$. The $MgATP^{2-}$ concentration was varied in a constant ratio of 1:1 with that of $MgIDP^-$. IDP^{3-} was maintained constant at 0.1 mM. Velocity is expressed as mμmoles of IMP per min per μg of protein.

carried out to determine if this property of the enzyme could be destroyed without affecting the catalytic activity of the enzyme. Heat treatment, assaying in the presence of $HgCl_2$, or assaying in high concentrations of urea all showed that the ability of the enzyme to be activated by $MgATP^{2-}$ was unimpaired as the catalytic activity was lost (Fig. I.14). Thus, when enzyme samples were assayed in both the presence and absence of $MgATP^{2-}$ under the conditions or after the treatment described above, the reaction rates were always faster in the presence of $MgATP^{2-}$.

When the purified enzyme was heat inactivated at 45°C both in the presence and absence of $MgATP^{2-}$, rapid inactivation resulted in both cases, although some protection was provided by the presence of a high (1.0 mM) concentration of $MgATP^{2-}$ (Fig. I.15). After the enzyme had been treated in this manner, the enzyme was still capable of being activated by $MgATP^{2-}$ (Insert, Fig. I.15).

The results of all the above inactivation studies are consistent with the catalytic activity of the enzyme being more susceptible to inactivation than the allosteric site.

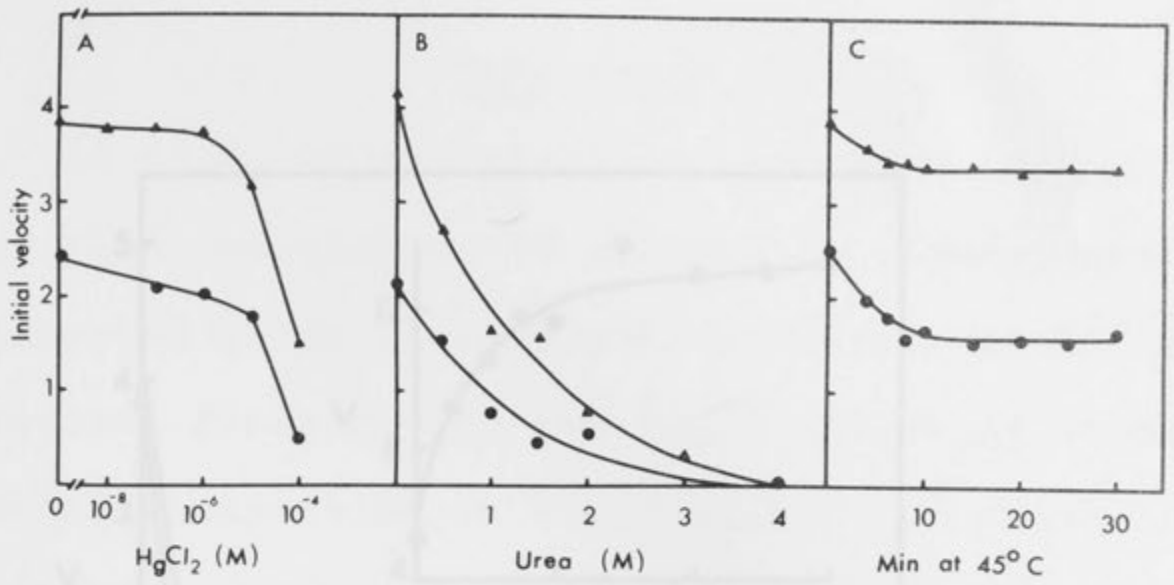


FIGURE I.14. Effect of denaturing agents on the catalytic and allosteric properties of nucleoside diphosphatase. Partially purified enzyme (0.75 mμM/min/μg protein) was assayed in triethanolamine, 0.1 M, pH 8.5, containing 10^{-5} M EDTA and 0.125 mM $MgIDP^-$. Assays were carried out both in the absence (●) and presence (▲) of 0.3 mM $MgATP^{2-}$ under the following conditions : (A) Enzyme (10 μg) was added to the reaction mixture which in addition contained the indicated amounts of $HgCl_2$. EDTA was omitted from the reaction mixture. Enzyme activity is expressed in arbitrary units. (B) Enzyme (10 μg) was added to the reaction mixtures, which in addition to the ingredients described above, contained the indicated amounts of urea. (C) An enzyme solution containing 5 mg/ml of protein was heated in 0.1 M Tris pH 8.0 which contained 10^{-5} EDTA and 10^{-4} dithiothreitol. Enzyme samples (10 μg) were removed for assaying at the indicated times.

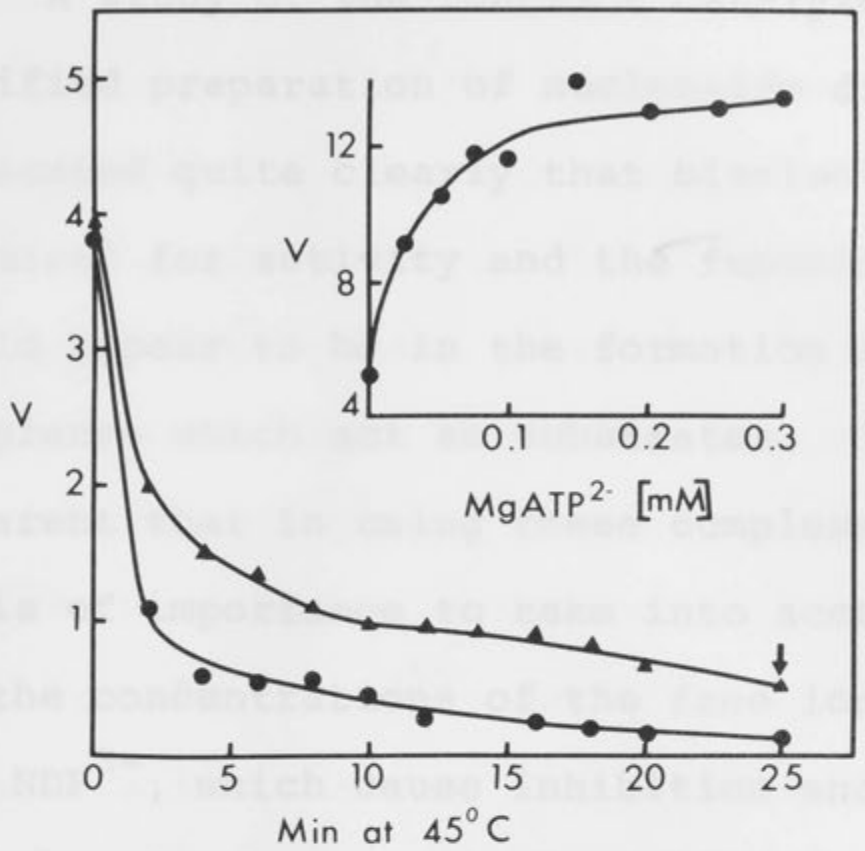


FIGURE I.15. Effect of heat on purified nucleoside diphosphatase in the presence and absence of $MgATP^{2-}$. The enzyme, 0.55 mg/ml was heated in 0.1 M triethanolamine, pH 8.5 in the presence (▲) and absence (●) of 1.0 mM $MgATP^{2-}$ for the indicated times. A sample of enzyme was removed at the indicated (⬇) time and used to determine the effect of $MgATP^{2-}$ on the initial velocity of the reaction at a fixed (0.4 mM) of $MgIDP^{-}$ (figure insert). Initial velocities are expressed in arbitrary units.

Discussion

A study of the reaction catalyzed by a highly purified preparation of nucleoside diphosphatase has indicated quite clearly that bivalent metal ions are required for activity and the function of these ions would appear to be in the formation of metal-nucleotide complexes which act as substrates. It has also become apparent that in using these complexes as substrates, it is of importance to take into account the variation in the concentrations of the free ionic species, Mg^{2+} and NDP^{3-}, which cause inhibition and activation of the reaction, respectively. Thus the nature of the kinetic results obtained can vary according to the equilibrium relationship that exists between the concentrations of the free and complex species.

Under the conditions in which the concentration of IDP^{3-} is held constant at a low concentration, plots of $1/v$ against $1/MgIDP^{-}$ are non-linear and have the form of a non-rectangular hyperbola which is concave up. Such a result has been shown to be in qualitative agreement with the interdependent reaction of two molecules of substrate with the enzyme to form E-MgIDP and $E\text{-}(MgIDP)_2$, both of which can break down to yield the products, IMP and inorganic phosphate.

Such an interdependent reaction could come about as a result of a conformational change in the enzyme following the addition of the first substrate molecule. In the presence of compounds that can be considered as allosteric activators, double reciprocal plots of initial velocity as a function of substrate concentration are linear and it has been suggested that this is due to the reaction of one molecule of activator at one of the two substrate sites. A more rigid test of whether or not the proposals are tenable lies in demonstrating that the initial velocity equation derived on the basis of the mechanism can adequately account for the kinetic data. The results of this approach, together with possible alternative random mechanisms, are reported in Chapter II. It should be noted that no comments have been made concerning the structure of the various enzyme forms with the exception of the fact that under the conditions tested, no polymerization or dissociation reactions could be detected. While detection of such enzyme forms is of importance with respect to an understanding of the overall reaction mechanism, no conclusions about the structure of intermediate species of enzyme can be drawn from kinetic data.

In contrast to the non-linear double reciprocal plots obtained using $MgIDP^-$ as the substrate, a study of the reaction velocity as a function of the concentrations of $MgUDP^-$ and $MnIDP^-$ gave rise to plots which were linear. These latter results could be interpreted to indicate that only one molecule of substrate can combine with the enzyme. However, they are not necessarily in disagreement with the reaction of two molecules of substrate, for if the binding of the first were strong, then over the range of concentrations used the results could approximate to those expected if only one molecule of substrate underwent reaction. Further, it is possible that free UDP^{3-} might be a better activator than other free NDP^{3-} species. A change in the relative binding of the two molecules of $MgIDP^-$ as a result of ageing the enzyme would also account for the linear kinetics obtained with this preparation. It is of interest to note that under the conditions which yield linear double reciprocal plots, there is still activation of the enzyme by $MgATP^{2-}$.

The ability of Mg^{2+}, Mn^{2+} or Ca^{2+} to function as the essential bivalent metal ion for the nucleoside diphosphatase reaction is reminiscent of the results obtained with other phosphotransferases such as

creatine kinase (Morrison and Uhr, 1966; O'Sullivan and Cohn, 1966) and in contrast to the finding that Ca^{2+} strongly inhibits pyruvate kinase (Kachmar and Boyer, 1953). On the basis of the hypothesis proposed by Cohn (1963), it would be concluded that nucleoside diphosphatase does not form an enzyme-metal complex. This conclusion is in accord with the postulate that the metal-nucleotide complexes act as substrates.

Although the substrate specificity of the enzyme is broad, only the magnesium complexes of IDP, UDP and GDP can be considered as good substrates. From a comparison of the reaction velocities and the structure of the nucleotide moiety of substrate, it appears that the 6-oxypurine group, which these three nucleotides have in common, is necessary for full catalytic activity. However, it remains to be determined if this structural feature is of importance with respect to the binding of the first substrate molecule or its ability, in the form of an enzyme-substrate complex, to enhance the reaction of the second molecule of substrate. Irrespective of whether or not a nucleotide is a good substrate, the rate at which it undergoes reaction is increased in the

presence of $MgATP^{2-}$. Further, it would seem that the magnesium complexes of the naturally occurring nucleoside triphosphates which function as allosteric activators, do not show any specificity for the purine or pyrimidine moiety (cf. Yamazaki and Hayaishi, 1965) although phosphonate analogues of ATP and inorganic triphosphates are ineffective as activators of the reaction.

The finding that the allosteric site of nucleoside diphosphatase cannot be destroyed apart from destruction of the catalytic activity of the enzyme, is in contrast to the findings of some other allosteric enzymes (e.g. Stadtman, 1966: Atkinson, 1966) in which the allosteric property is readily destroyed. The observed results for nucleoside diphosphatase are ambiguous in that no definite conclusions can be reached, except that they are consistent with the catalytic activity being more liable to destruction than the allosteric site.

Summary

Nucleoside diphosphatase from rat liver has been purified over 800-fold and used for preliminary kinetic studies of the metal ion activation, substrate specif-

icity and mechanism of action of the enzyme, as well as of its allosteric activation by nucleoside triphosphates. The essential divalent metal ion requirement was met by Mg^{2+}, Mn^{2+} or Ca^{2+} and it appears that these ions are involved in the formation of metal-nucleotide complexes that function as substrates for the reaction. At pH 8.5 and a concentration of 0.5 mM, the rate of hydrolysis of the magnesium complexes of various nucleoside diphosphates, and thiamine pyrophosphate decreased in the order : uridine diphosphate ≈ guanosine diphosphate ≈ inosine diphosphate >> cytosine diphosphate > thiamine pyrophosphate >> adenosine diphosphate. No hydrolysis of d-thymidine diphosphate was detected. When $MgIDP^-$ was used as the substrate and IDP^{3-} maintained at a concentration of 0.1 mM, double reciprocal plots of the initial velocity as a function of substrate concentration were curvilinear. Relatively high concentrations of Mg-nucleoside triphosphates or IDP^{3-} had the effect of increasing the initial reaction velocity at lower substrate concentrations with the result that the double reciprocal plots became linear. It was concluded that the above results were consistent with the idea that while two molecules of substrate can undergo interdependent reaction with the enzyme, the

addition of only one is necessary for reaction to occur. From the data relating to the activating effects of Mg-nucleoside triphosphates and IDP^{3-}, it has been concluded that these allosteric activators combine with the enzyme at one of the sites at which $MgIDP^{-}$ can react. Higher concentrations of free Mg^{2+} have been found to inhibit the reaction.

CHAPTER II

KINETIC STUDIES OF THE MECHANISM AND ALLOSTERIC ACTIVATION OF THE REACTION CATALYZED BY NUCLEOSIDE DIPHOSPHATASE

CHAPTER II

KINETIC STUDIES OF THE MECHANISM AND ALLOSTERIC ACTIVATION OF THE REACTION CATALYZED BY NUCLEOSIDE DIPHOSPHATASE

Introduction

At the present time, no general conclusions can be reached about the mechanism of action of allosteric enzymes. The majority of kinetic investigations have been primarily qualitative and thus suffer from a number of weaknesses. Prime among these is the failure to obtain sufficient experimental data so that an analysis can be made by fitting the data to a particular velocity equation. Computer programs have been developed for the fitting of data to two such velocity equations (Cleland, 1967) which were derived on the basis of classical kinetic theory, but as discussed in the Introduction, they have had only limited use (Worcel et al., 1965; Sanwal et al., 1965; Sanwal and Cook, 1966).

It has been more usual to attribute the results of kinetic studies with allosteric enzymes to their existence in isomeric forms as postulated by Monod et al. (1965) and Koshland et al. (1966) or to their

ability to undergo polymerization reactions as proposed by Nichol et al. (1967). These hypotheses assume that the kinetically observed allosteric data are due solely to the thermodynamic effects of enzyme-substrate interactions and give no consideration to the possibility that they are due to changes in the rates of product formation from different enzyme-substrate complexes. Thus it was of interest to undertake a detailed investigation of a reaction catalyzed by an allosteric enzyme to ascertain if the resulting data could be explained in terms of a steady-state kinetic theory (cf. Frieden, 1964, 1967) for which there was no necessity to postulate the nature of the enzyme-substrate complexes.

The results reported in Chapter I suggested that the non-linear double reciprocal plots of initial velocity against $MgIDP^-$ concentration could be accounted for on the basis of the interdependent reaction of two molecules of $MgIDP^-$ at two active sites on the enzyme. But since the above results were only qualitative and do not constitute proof of the proposed hypothesis, it was of importance to determine if there was quantitative agreement

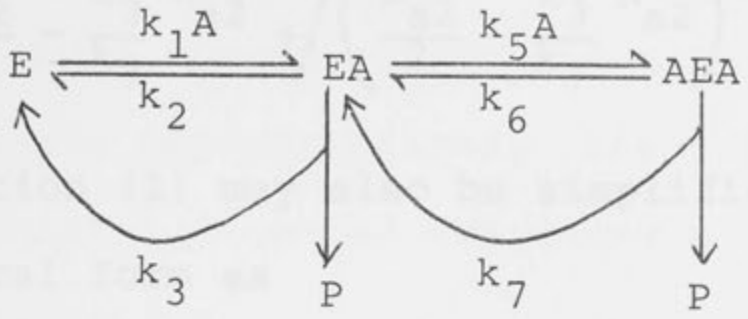
between the proposed scheme and the experimental data. For this purpose, appropriate initial velocity equations have been derived and used in connection with the analysis of the initial velocity data. From such analyses it has been shown that the data are consistent with the interdependent combination of two molecules of substrate, but that only one is needed before catalytic reaction can occur. The data are also consistent with the combination of one molecule of allosteric activator at one of the catalytic sites. Values have been obtained for the kinetic constants associated with both substrate and modifier reaction.

Theory

Kinetics of the reaction in the absence of modifier. As will be discussed later, there are a number of mechanisms that are in accord with the present kinetic results since they yield the same general form of initial velocity equation. But for the purpose of illustrating the form and the general characteristics of the equation as well as fitting the experimental data, a choice has been made of the simplest ordered steady-state mechanism which can account for the data and permits determination of the

various kinetic constants. The mechanism involves the reaction of two molecules of substrate which is the minimum requirement to account for the type of kinetic data obtained with a number of allosteric enzymes when it is considered that the native enzyme exists only as a single molecular species (cf. Weber and Anderson, 1965). Thus it is envisaged that one substrate molecule (A) reacts at an active site on the native enzyme (E) to form an EA complex which can give rise to products (P). It is further assumed that an additional molecule of substrate reacts with the EA complex at a second site, either pre-existing on the native enzyme or formed as a consequence of EA formation, to give an AEA complex which also breaks down to yield products. No assumptions are made about the relative rates of combination of A with the E and EA complexes, or about the rates of product formation from EA and AEA. However, it is assumed that the breakdown of the AEA complex yields products and the EA complex which in turn breaks down to products and free enzyme.

The reaction sequence can be written as :



and the initial velocity equation as the ratio of two polynomials viz :

$$v = \frac{V\left(A^2 + \frac{k_3}{k_7} K_{a2}A\right)}{A^2 + K_{a2}A + K_{a1}K_{a2}} \qquad (1)$$

where $V = k_7 e_t$, $K_{a1} = \frac{k_2 + k_3}{k_1}$ and $K_{a2} = \frac{k_6 + k_7}{k_5}$. The kinetic constants K_{a1} and K_{a2} will be called combination constants in order to distinguish them from Michaelis constants which they resemble in form but from which they differ in that they do not represent the concentration of substrate required to give half maximum velocity. Combination constants are similar to Michaelis constants for single substrate reactions in being composed of both a thermodynamic and kinetic element. It follows from equation (1) that the Michaelis constant (K_m) is equal to

$$\frac{K_{a2}}{2} - \frac{k_3}{k_7} K_{a2} + \sqrt{\left(\frac{K_{a2}}{2} - \frac{k_3}{k_7} K_{a2}\right)^2 + K_{a1}K_{a2}}$$

Equation (1) may also be simplified by writing it in general form as

$$v = \frac{V(A^2 + dA)}{A^2 + bA + c} \qquad (2)$$

where $d = \frac{k_3}{k_7} K_{a2}$, $b = K_{a2}$ and $c = K_{a1}K_{a2}$ (Cleland, 1967). The initial velocity expression (1) is an equation of a curve such that plots of v against A would have a sigmoidal shape and a positive initial slope. The latter conclusion is reached as a result of differentiating equation (1) and setting A = 0 when it is found that the initial slope is equal to

$$\frac{k_3 V}{k_7 K_{a1}} \quad \text{or} \quad \frac{k_3 e_t}{K_{a1}} .$$

When written in reciprocal form, equation (1) becomes

$$\frac{1}{v} = \frac{K_{a1}K_{a2}\left(\frac{1}{A}\right)^2 + K_{a2}\left(\frac{1}{A}\right) + 1}{V\left[\frac{k_3}{k_7} K_{a2}\left(\frac{1}{A}\right) + 1\right]} \qquad (3)$$

which Cleland (1963b) refers to as a 2/1 function. Thus plots of 1/v against 1/A give non-rectangular hyperbolas which are characterized by having an

asymptote in the region of low substrate concentrations and a curved portion in the region of high substrate concentrations. The vertical intercept and initial slope of the curve would be given by the relationships

$$\underset{[Int(1)]}{\text{vertical intercept}} = \frac{1}{V} \quad \text{and} \quad \underset{[S1(1)]}{\text{initial slope}} = \frac{K_{a2}}{V}\left(1 - \frac{k_3}{k_7}\right) \qquad (4)$$

respectively. Thus the initial slope of the curve could be positive, zero or negative, depending on the values of the rate constants. The extrapolated vertical intercept and slope of the asymptote would be given by the relationships

$$\underset{[Int(2)]}{\text{Extrapolated intercept of asymptote}} = \frac{1}{V}\left(1 - \frac{k_7}{k_3}\frac{K_{a1}}{K_{a2}}\right)\frac{k_7}{k_3} \quad \text{and} \qquad (5)$$

$$\underset{[S1(2)]}{\text{Slope of asymptote}} = \frac{k_7}{k_3}\frac{K_{a1}}{V} = \frac{K_{a1}}{k_3 e_t},$$

from which it is apparent that while the slope of the asymptote must always be positive, the intercept may have a positive, zero or negative value, depending on the ratios of the rate and kinetic constants.

The relationships given in (4) and (5) are also of importance with respect to the determination of estimates for the kinetic constants and the ratio of rate constants. It may be shown that

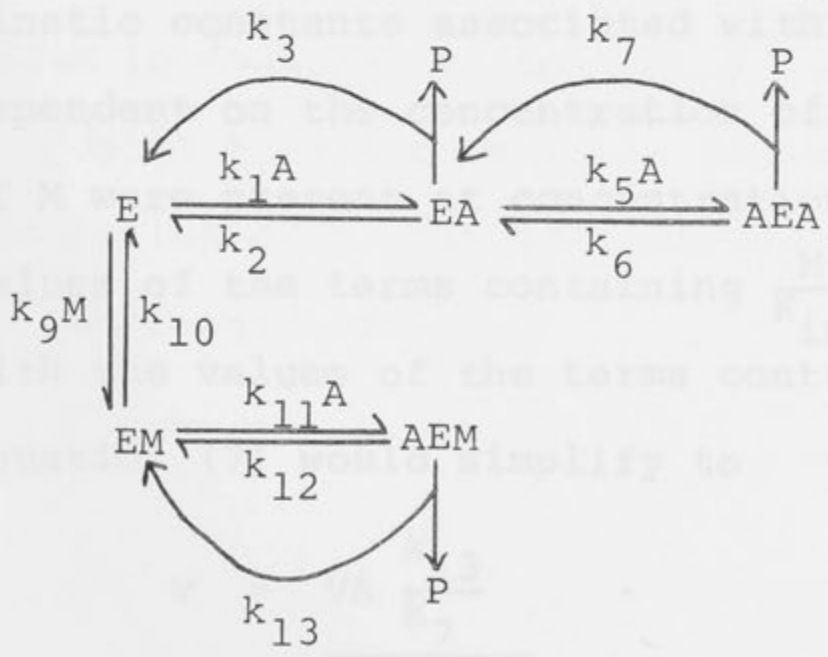
$$\frac{Sl(1) - Sl(2)}{Int(2) - Int(1)} = \frac{k_3}{k_7} K_{a2} = d \qquad (6a)$$

$$\frac{Sl(2) \times d}{Int(1)} = K_{a1} K_{a2} = c \qquad (6b)$$

$$\text{and} \quad d + \frac{Sl(1)}{Int(1)} = K_{a2} = b \qquad (6c)$$

where d, c and b correspond to the parameters of the general equation (2). It follows then that values may be calculated for K_{a1}, K_{a2} and $\frac{k_3}{k_7}$, in addition to the maximum velocity (V). However, it has been shown (Kowalik and Morrison, 1968) that the values obtained by graphical analysis are subject to considerable error because of the difficulty of drawing the curve of best fit to the experimental points. Further, it has been pointed out in connection with the quantitative analysis of data that initial velocity values over a wide range of substrate concentrations are required to obtain reasonable estimates of the slopes and intercepts of the curve (relationships given in 4 and 5), as well as to define its shape.

Kinetics of the reaction in the presence of modifier. It is considered that a modifier (M), of similar structure to that of the substrate, is capable of reacting with the native enzyme at the site where the first molecule of substrate combines so as to form an EM complex. In addition, it is assumed that this complex cannot undergo further reaction with the modifier, because of the resultant conformational change at the second site, but can react with substrate to form an AEM complex which gives rise to products. However, the rates of formation and breakdown of the AEM and AEA complexes are not necessarily equal. The complete series of reactions can be illustrated as



and the initial velocity equation in the presence of

modifier written as

$$v = \frac{V\left(A^2 + \frac{k_3}{k_7} K_{a2} A + \frac{k_{13}}{k_7} \frac{K_{a1} K_{a2}}{K_A K_{im}} MA\right)}{A^2 + K_{a2} A + \frac{K_{a1} K_{a2}}{K_A K_{im}} MA + \frac{K_{a1} K_{a2}}{K_{im}} M + K_{a1} K_{a2}} \qquad (7)$$

where, in addition to the kinetic constants previously defined,

$$K_A = \frac{k_{12} + k_{13}}{k_{11}} \quad \text{and} \quad K_{im} = \frac{k_{10}}{k_9}$$

For the condition that M is present at a fixed, non-saturating concentration, plots of v against A and 1/v against 1/A would have the same general characteristics as those described above since, under these conditions, equations (7) and (1) have the same form. But only apparent values would be obtained directly for the kinetic constants associated with A as they would be dependent on the concentration of M. On the other hand, if M were present at concentrations such that the values of the terms containing $\frac{M}{K_{im}}$ were large compared with the values of the terms containing only A, then equation (7) would simplify to

$$v = \frac{VA \frac{k_{13}}{k_7}}{K_A + A} \qquad (8)$$

so that a plot of 1/v against 1/A would be linear with the horizontal intercept giving a measure of K_A. It should be noted, however, that the above condition does not hold when the concentration of A is high relative to the kinetic constants associated with A. Thus in this type of experiment the vertical intercept would give a measure of the maximum velocity for the breakdown of the AEA, rather than the MEA complex. At the vertical intercept, the concentration of A would be infinite compared with a finite concentration of M and as M and A are assumed to react with the same form of enzyme (E), M would be displaced leaving only the AEA form. To determine the rate of AEM breakdown, it would be necessary to use M as the variable reactant at different fixed substrate concentrations or to vary A and M in constant ratio.

With M as the variable reactant at a fixed concentration of A, the initial velocity equation (7) may be rearranged as

$$v = V\left[\frac{A^2 + \frac{k_3}{k_7} K_{a2}A}{A^2 + K_{a2}A + K_{a1}K_{a2}}\right] \quad X$$

$$\left[\frac{1 + \left(A^2 + \frac{k_3}{k_7} K_{a2}A\right)\left(\frac{k_7 K_A K_{im}}{k_{13} K_{a1} K_{a2}}\right)^{M}}{1 + \left(A^2 + K_{a2}A + K_{a1}K_{a2}\right)\left(\frac{K_A K_{im}}{K_{a1}K_{a2}(K_A + A)}\right)^{M}}\right] \tag{9}$$

The general form of this equation is

$$v = V'\left\{\frac{1 + \frac{M}{K_{iN}}}{1 + \frac{M}{K_{iD}}}\right\} \tag{10}$$

where $V' = V\left\{\frac{A^2 + \frac{k_3}{k_7} K_{a2}A}{A^2 + K_{a2}A + K_{a1}K_{a2}}\right\}$ (11)

$$K_{iN} = \left(A^2 + \frac{k_3}{k_7} K_{a2}A\right)\left(\frac{k_7 K_A K_{im}}{k_{13} K_{a1} K_{a2}}\right) \tag{12}$$

and $K_{iD} = \left(A^2 + K_{a2}A + K_{a1}K_{a2}\right)\left\{\frac{K_A K_{im}}{K_{a1}K_{a2}(K_A + A)}\right\}$ (13)

so that plots of v against M would yield rectangular hyperbolas that do not pass through the origin. Values for the complex constants, K_{iN} and K_{iD}, can be determ-

ined by means of computer analysis of the experimental data. Substitution into the relationship for K_{iD} (equation 13) of values for K_{a1}, K_{a2} and K_A would yield the true value for K_{im} which can be used in the determination of the $\frac{k_{13}}{k_7}$ ratio from the relationship given for K_{iN} (equation 12).

Alternatively, values for K_{im} and the $\frac{k_{13}}{k_7}$ ratio may be determined from the apparent kinetic constants obtained from a linear plot of $1/v-v_o$ against $1/M$ where v and v_o represent the initial velocities in the presence and absence of modifier, respectively. The horizontal intercept of such a plot would yield an apparent K_{im} value from which the true K_{im} value could be calculated using the relationship

$$K_{im} = \text{apparent } K_{im}\left[\frac{K_{a1}K_{a2}\left(1 + \frac{A}{K_A}\right)}{A^2 + K_{a2}A + K_{a1}K_{a2}}\right] \qquad (14)$$

while the vertical intercept would be represented by the expression

$$\frac{1}{V}\left[\frac{K_A + A}{\frac{k_{13}}{k_7}\frac{\left(A^2 + \frac{k_3}{k_7}K_{a2}A\right)\left(K_A + A\right)}{A^2 + K_{a2}A + K_{a1}K_{a2}}}\right] \qquad (15)$$

which can be used to determine the $\frac{k_{13}}{k_7}$ ratio.

The initial velocity equation when the modifier M and the substrate concentrations are varied in constant ratio becomes

$$v = \frac{V\left[\dfrac{K_A K_{im} + \dfrac{k_{13}}{k_7} K_{a1} K_{a2} x}{K_A K_{im} + K_{a1} K_{a2} x}\right]\left[A^2 + \dfrac{\dfrac{k_3}{k_7} K_A K_{im} K_{a2}}{K_A K_{im} + \dfrac{k_{13}}{k_7} K_{a1} K_{a2} x} A\right]}{A^2 + \dfrac{K_A K_{a2}\left(K_{im} + K_{a1} x\right)}{K_A K_{im} + K_{a1} K_{a2} x} A + \dfrac{K_A K_{im} K_{a1} K_{a2}}{K_A K_{im} + K_{a1} K_{a2} x}} \qquad (16)$$

where $M = xA$. When equation (16) is written in general form as

$$v = \frac{V'(A^2 + d'A)}{A^2 + b'A + c'} \qquad (17)$$

where $V' = V\left[\dfrac{K_A K_{im} + \dfrac{k_{13}}{k_7} K_{a1} K_{a2} x}{K_A K_{im} + K_{a1} K_{a2} x}\right]$ (18),

$$d' = \frac{\dfrac{k_3}{k_7} K_A K_{im} K_{a2}}{K_A K_{im} + \dfrac{k_{13}}{k_7} K_{a1} K_{a2} x} \qquad (19),$$

$b' = \dfrac{K_A K_{a2}\left(K_{im} + K_{a1} x\right)}{K_A K_{im} + K_{a1} K_{a2} x}$ (20), $\quad c' = \dfrac{K_A K_{im} K_{a1} K_{a2}}{K_A K_{im} + K_{a1} K_{a2} x}$ (21)

it becomes apparent that it is also a 2/1 function with the same characteristics as those described for equations (1) and (2).

The individual values for V', b', c' and d' may be obtained by analysis of the data and then substituted, together with the values for V, K_A, K_{a1}, K_{a2}, K_{im} and x into equations (18-21), to determine the value for $\frac{k_{13}}{k_7}$. It should be noted that the directly determined apparent maximum velocity, V', is dependent upon the relative rates of breakdown of the MEA (k_{13}) and AEA (k_7) complexes, as well as the values for all the kinetic constants.

Experimental

Materials and Methods

All materials and the methods for estimating the reaction velocity have been described in Chapter I. Particular care was taken to ensure that initial reaction rates were measured since the failure to determine initial velocities at the lower concentrations of substrate would result in misleading, non-linear double reciprocal plots of velocity as a function of substrate concentration. The calculations of weighted mean values of the kinetic constants, to-

gether with their standard errors, were made as elaborated by Morrison and Uhr (1966) where :

$$\text{Weighted mean of x values} = \frac{\Sigma WiXi}{\Sigma Wi}$$

and

$$\text{S.E. of weighted mean value} = \frac{1}{\sqrt{\Sigma Wi}}$$

$$\text{where } Wi = \frac{1}{[\text{S.E. } Xi]^2}$$

and the standard error of sums, products and quotients are given by :

$$\text{S.E. } (x + y) = \sqrt{(\text{S.E.}(x))^2 + (\text{S.E.}(y))^2}$$

$$\text{S.E. } (xy) = xy\sqrt{\left(\frac{\text{S.E.}(x)}{x}\right)^2 + \left(\frac{\text{S.E.}(y)}{y}\right)^2}$$

$$\text{S.E. } \left(\frac{x}{y}\right) = \frac{x}{y}\sqrt{\left(\frac{\text{S.E.}(x)}{x}\right)^2 + \left(\frac{\text{S.E.}(y)}{y}\right)^2}$$

Analysis of data. The type of curve obtained was determined by graphical analysis of the data which were then analyzed by an appropriate computer program. Data conforming to a linear, non-competitive inhibition pattern or to equations (8) and (9) were analyzed using the Fortran programs of Cleland (1963d). Data conforming to equation (1) were analyzed by the computer

programs of both Cleland (1963d) and Kowalik and Morrison (1968) which were modified so as to allow for the inclusion of weighting factors (see Results). In connection with the use of these programs, it should be mentioned that it is first necessary to plot the experimental data in double reciprocal form so that estimates can be made of the initial slope of the curve, the slope of the asymptote, the intercept of the curve and the extrapolated intercept of the asymptote (see Theory). The values so obtained provide starting values for the non-linear analysis. Because the program of Cleland (1963d) utilizes a Gaussian method, reasonably accurate estimates of the above parameters are required, whereas this is not the case with the gradient minimization method used by Kowalik and Morrison (1968). Since these estimates are sometimes difficult to obtain, the procedure adopted was to make a preliminary fit of the data by means of the Kowalik and Morrison program and to use the output of refined estimates from this analysis as the starting point for the final analysis by the Cleland program. With good starting values, both programs gave the same minimum value for the residual sum of squares. All analyses

were performed on an I.B.M. 360 computer, and the values of the constants so obtained were used in the drawing of the lines for the figures.

Results

Initial velocity studies and analysis of data.

In the first series of kinetic experiments, initial velocities were determined over a relatively narrow range of substrate concentrations. It was found that the data did not give good fits to equation (1) when analyzed by means of the computer program described by Kowalik and Morrison (1968), and the standard errors of the kinetic parameters were high. This was presumed to be due to an insufficient number of points to define the shape of the curve and further investigations were made by determining a large number of initial rates over a wide (50-fold) range of $MgIDP^-$ concentrations. Analysis of such data showed that for a plot of velocity against substrate concentration (Figure II.1A), there appeared to be good agreement between the theoretical and experimental points. However, when the same data were plotted in double reciprocal form, it became apparent that the theoretical curve did not pass through the experimental points at low substrate con-

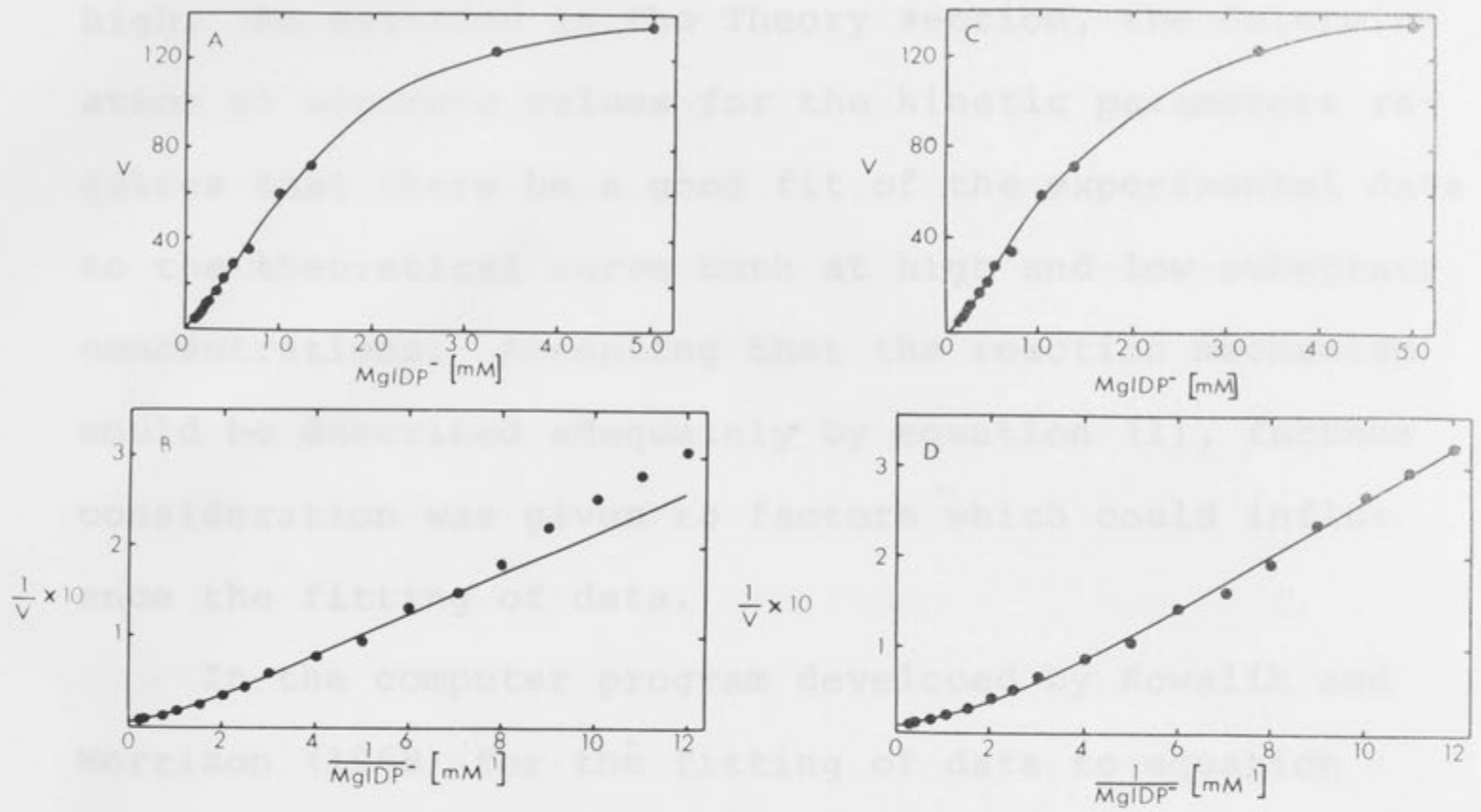


FIGURE II.1. The effect of making unweighted (A and B) and weighted (C and D) fits of initial velocity data to equation (2). The weighting factor used was $1/v^2$ where v is the initial velocity. The lines represent the theoretical curves which were drawn by using the constants obtained from the analyses and v is expressed as mμmoles per min per μg of protein.

centrations (Figure II.1B). Furthermore, the standard errors of some of the kinetic constants were relatively high. As outlined in the Theory section, the determination of accurate values for the kinetic parameters requires that there be a good fit of the experimental data to the theoretical curve both at high and low substrate concentrations. Accepting that the reaction mechanism could be described adequately by equation (1), further consideration was given to factors which could influence the fitting of data.

In the computer program developed by Kowalik and Morrison (1968) for the fitting of data to equation (1), no weighting factors were incorporated since the usual assumption was made that the variance associated with the velocity determinations was constant. If this assumption were not valid and the variance increased as a function of the velocity, then for a wide range of values, the contribution made by the lower velocities to the residual sum of squares of a least squares fit of the data would be negligible compared with that of the higher velocities. Consequently, they would have little influence in defining the shape of the curve, as well as the values for the kinetic parameters, and a plot such as that shown in Figure II.1B could be

obtained. Parenthetically, it should be mentioned that this situation differs from that which appertains to the study of reactions conforming to Michaelis-Menten kinetics where only a very limited range of velocities for a single curve is normally encountered.

It was, therefore, of importance in connection with the analysis of kinetic data for the nucleoside diphosphatase reaction to establish the relationship between the variance and the magnitude of the reaction velocities. The results of such experiments (Table II.1) showed that the standard deviation (square root of the variance) of the mean velocity values at four different substrate concentrations was proportional to the velocity value. Thus there was justification for making a weighted least squares fit of the data to equation (1) by the inclusion in the computer program of weighting factors equal to the reciprocal of the square of each initial velocity (Cleland, 1967). This results in each velocity value making an equal contribution to the residual sum of squares and reanalysis of the data illustrated in Figure II.1 (A and B) demonstrated the good agreement that is now obtained between the theoretical curve and the experimental points (Figure II.1, C and D). It may be concluded

TABLE II.1. : Standard deviations of mean velocity values at four different substrate concentrations.

Substrate Concentration (mM)	Initial Velocity [a]	Standard Deviation	Standard Deviation (%)	Number of Determinations
0.1	10.8	0.35	3.2	10
0.3	32.6	1.01	3.1	10
1.5	119	3.58	3.0	10
5.0	179	5.80	3.2	9

[a] Velocities were not corrected for inhibition by free Mg^{2+} and are expressed as mμmoles per min per μg of protein.

that the kinetic results are in accord with the proposed mechanism for the reaction. In view of the above finding, all subsequent analyses of data, conforming to equations (1), (7) or (16) were made by weighted least squares fitting as described under Methods.

Determination of kinetic constants. For the estimation of the maximum velocity of the reaction and of the combination constants, K_{a1} and K_{a2}, as well as the ratio of the rate constants, k_3/k_7, a series of experiments were carried out in which the initial velocity of the reaction was determined at 22 different substrate concentrations within the range from 0.1 to 5.0 mM. A typical result is illustrated in Figure II.2 (A and B) while the weighted mean values for the above parameters from four experiments are listed in Table II.2. The fit of the curve to the experimental data and the values for the kinetic constants confirm that the results are in accord with those predicted by equation (1). Moreover the data did not give good fits to an equation for a parabola such as would be obtained when the d term of equation (2) was zero (Cleland, 1963d). It should be noted that, on extrapolation, the asymptote of Figure II.2B will cut the

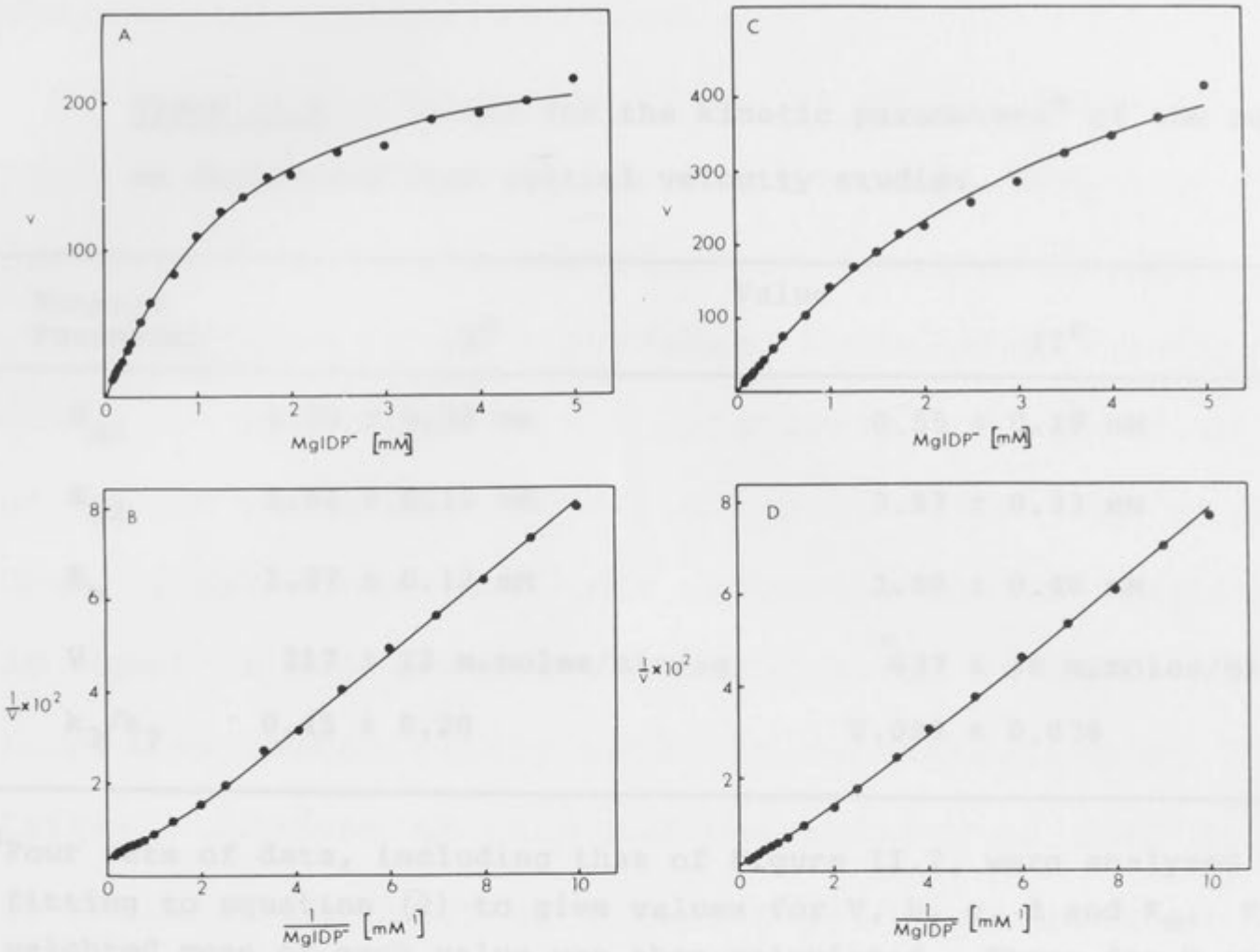


FIGURE II.2. The effect of the concentration of $MgIDP^-$ on the initial velocity of the reaction before (A and B) and after (C and D) correction for the inhibition by free Mg^{2+}. The concentration of free IDP^{3-} was held constant at 0.1 mM and corrections for the inhibition by free Mg^{2+} were made as described in Table II.2 using the constants given in Table II.3. A weighted fit of the data to equation (2) was made and the constants so obtained were used to draw the theoretical curves which are represented by the solid lines. v is expressed as mμmoles per min per μg of protein.

TABLE II.2. : Values for the kinetic parameters[a] of the reaction as determined from initial velocity studies.

Kinetic Parameter	Value I[b]	Value II[c]
K_{a1}	1.00 ± 0.38 mM	0.55 ± 0.19 mM
K_{a2}	1.61 ± 0.12 mM	3.97 ± 0.33 mM
K_m	1.27 ± 0.12 mM	3.80 ± 0.40 mM
V	217 ± 12 mμmoles/min/μg	637 ± 38 mμmoles/min/μg
k_3/k_7	0.45 ± 0.20	0.083 ± 0.036

[a]Four sets of data, including that of Figure II.2, were analyzed by fitting to equation (2) to give values for V, b, c, d and K_m. The weighted mean of each value was then calculated. Those for V and K_m are recorded above while those for b, c and d were 1.61 ± 0.12, 1.61 ± 0.60 and 0.725 ± 0.317 mM, respectively, before correction and 3.97 ± 0.33, 2.17 ± 0.72 and 0.329 ± 0.142 mM, respectively, after correction for inhibition by free Mg^{2+}. These latter values were used in conjunction with the relationships (6a-6c) to determine values for K_{a1}, K_{a2} and the k_3/k_7 ratio.

[b]Values obtained by analysis of data which had not been corrected for the inhibition by free Mg^{2+}.

[c]Values obtained by analysis of data after correction for free Mg^{2+} (I) inhibition using the relationship :

$$\text{true velocity} = \text{apparent velocity} \Big/ \left\{ \frac{K + A}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{iI}}\right)} \right\}$$

and the values for K, K_{is} and K_{iI} given in Table II.3.

ordinate at a negative value.

Inhibition of the reaction by Mg^{2+}. Since it has been shown that, at lower substrate concentrations, free IDP^{3-} activates the reaction (Chapter I), the above experiments were performed by holding the free IDP^{3-} concentration at the relatively low concentration of 0.1 mM while the concentration of $MgIDP^{-}$ was varied. Under these conditions, the concentration of free Mg^{2+} is equal to 2.5 times the concentration of $MgIDP^{-}$ and increases concomitantly with the concentration of the latter. Because of this and the ability of free Mg^{2+} to inhibit the reaction (Chapter I), true reaction velocities would not be measured at the higher concentrations of $MgIDP^{-}$. While it appears that the inhibitory action of free Mg^{2+} does not alter the form of rate equation (1), as judged by the results of Figure II.2B, it would influence the curved portion of double reciprocal plots and the values of the kinetic constants obtained by direct analysis of the data would be erroneous. Determination of the true values for these constants requires a knowledge of the inhibition constants associated with free Mg^{2+}, but because of the difficulty of carrying out experiments which would yield accurate values, a simpler approach was used to

obtain approximate values. Thus the inhibition by different fixed concentrations of free Mg^{2+} was studied over a small range of relatively high substrate concentrations where double reciprocal plots could be considered to approximate to straight lines. The results (Figure II.3A) demonstrate that linear plots are obtained and that appreciable inhibition occurs at levels of free Mg^{2+} encountered with $MgIDP^-$ concentrations from 1.0 to 5.0 mM.

For comparative purposes, a similar experiment was done to determine the apparent inhibition constants for free Mg^{2+} when the modifier, $MgATP^{2-}$, was present and varied in constant ratio with the substrate. It is apparent (Figure II.3B) that less inhibition is obtained under these conditions, but nevertheless, it is sufficient to require correction of the velocity values, especially at higher substrate concentrations. The kinetic constants obtained from analysis of the data in Figure II.3 are listed in Table II.3, while the method used to calculate uninhibited reaction velocities at any given concentrations of free Mg^{2+} and $MgIDP^-$ is elaborated in Table II.2. In practice, all the directly determined velocities were corrected by multiplying by factors. These ranged from 1.94-1.04

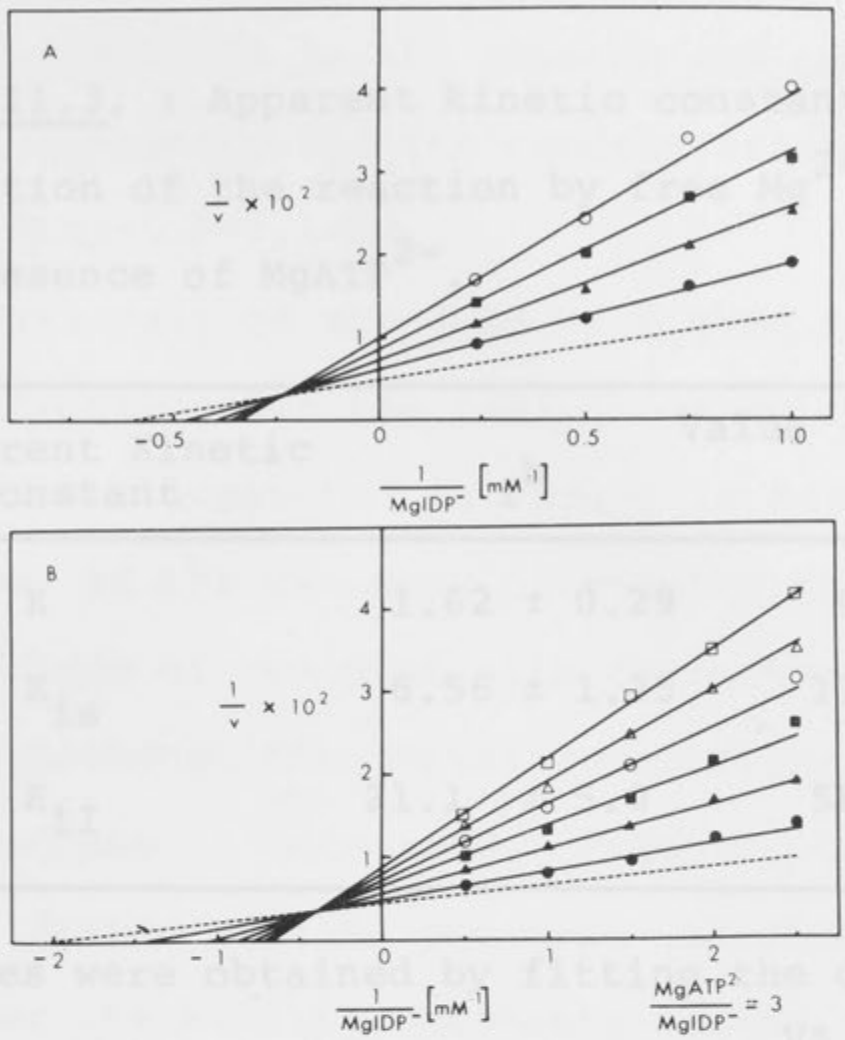


FIGURE II.3. Inhibition of the reaction by free Mg^{2+} in the absence (A) and presence (B) of $MgATP^{2-}$. For (B) the concentrations of $MgIDP^-$ and $MgATP^{2-}$ were varied simultaneously while maintaining their ratio constant. The concentrations of free Mg^{2+} for (A) were : 5 (●), 10 (▲), 15 (■) and 20 mM (o) while the concentrations for (B) were : 5 (●), 15 (▲), 25 (■), 35 (o), 45 (Δ) and 55 mM (□). The dashed lines represent the theoretical plots that would be obtained at zero concentration of free Mg^{2+}. The data were fitted to the equation given in the legend of Table II.3. v is expressed as mμmoles per min per μg of protein.

TABLE II.3. : Apparent kinetic constants[a] for the inhibition of the reaction by free Mg^{2+} in the absence and presence of $MgATP^{2-}$.

Apparent Kinetic Constant	Value I[b]	Value II[c]
K	1.62 ± 0.29	0.51 ± 0.05
K_{is}	6.56 ± 1.25	11.9 ± 1.4
K_{iI}	21.1 ± 5.0	58.5 ± 8.9

[a]Values were obtained by fitting the data of Figure II.3 to the equation : $$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{iI}}\right)}$$ where A and I represent concentrations of $MgIDP^-$ and free Mg^{2+}, respectively. Values are expressed as mM.

[b]$MgATP^{2-}$ was absent.

[c]Concentrations of $MgATP^{2-}$ and $MgIDP^-$ were varied in constant ratio.

and from 1.29-1.02 in the absence and presence of $MgATP^{2-}$, respectively, for concentrations of $MgIDP^{-}$ varying from 5.0-0.1 mM.

Re-analysis of the data of Figure II.2 (A and B) after correction for the inhibition by free Mg^{2+} gave the results illustrated in Figure II.2 (C and D). Comparison of the two sets of results shows that the general shape of the plot with corrected data is unchanged, although the curvature at higher substrate concentrations is decreased. Further, the data can still be fitted well to equation (1) to give the true values for the kinetic constants listed in Table II.2. It will be noted that the rate of breakdown of the MgIDP-enzyme-MgIDP complex (k_7) is about 12 times faster than the rate at which the enzyme-MgIDP complex gives rise to products (k_3). This difference may also be reflected in the values for the combination constants, K_{a1} and K_{a2}, which are determined by the relative rates of formation and breakdown of the enzyme-MgIDP and the MgIDP-enzyme-MgIDP complexes, respectively. Thus the value of K_{a1} is about 7 times less than that of K_{a2}.

Kinetics of the activation of the reaction by $MgATP^{2-}$ and IDP^{3-}. It has been demonstrated previously (Yamazaki and Hayaishi, 1965; Chapter I) that there is

activation of the reaction by $MgATP^{2-}$ and IDP^{3-} and that in the presence of sufficiently high concentrations of these nucleotide species, linear double reciprocal plots of velocity as a function of substrate concentrations are obtained. More detailed investigations of the effect of $MgATP^{2-}$ and IDP^{3-} on the initial velocity of the reaction at three different substrate concentrations gave the results illustrated in Figure II.4. Those of Figure II.4 (A and C) show that each set of experimental data gives a good fit to equation (10) and thus it may be concluded that only one molecule of $MgATP^{2-}$ or IDP^{3-} undergoes reaction with the enzyme. Analysis of the data yielded values for K_{iN} and K_{iD} which on substitution into equations (12) and (13), together with the values for K_{a1}, K_{a2}, K_A and k_3/k_7 gave values for the dissociation constant (K_{im}) of the enzyme-modifier complex, as well as for the relative rates of breakdown of the MgIDP-enzyme-modifier (k_{13}) and MgIDP-enzyme-MgIDP (k_7) complexes. The results obtained with $MgATP^{2-}$ and IDP^{3-} as modifiers are listed in Tables II.4 and II.5, respectively.

The data of Figure II.4A were also analyzed by an alternative procedure which involves the plotting of $1/v\text{-}v_o$, where v and v_o are initial velocities in the

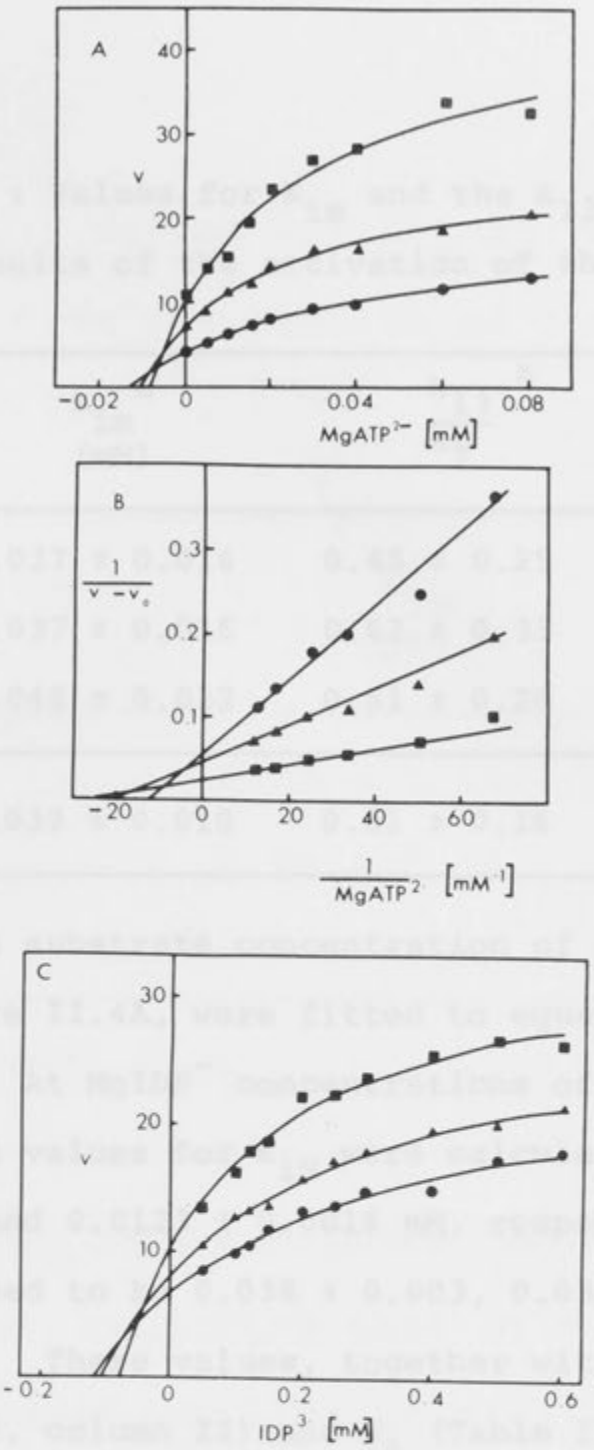


FIGURE II.4. Effect of the concentrations of $MgATP^{2-}$ and free IDP^{3-} on the initial velocity of the reaction at various fixed substrate concentrations. The concentrations of the substrate, $MgIDP^{-}$, were : 0.1 (●), 0.125 (▲) and 0.167 mM (■). The lines in (A), (B) and (C) were drawn using the constants obtained by fitting the data to equations (10), (8) and (10), respectively. In (B), v and v_o represent initial velocities in the presence and absence of $MgATP^{2-}$, respectively. All velocities are expressed as mμmoles per min per μg of protein.

TABLE II.4. : Values for K_{im} and the k_{13}/k_7 ratio as determined from the results of the activation of the reaction by $MgATP^{2-}$.

Concentration of $MgIDP^-$ (mM)	K_{im} [a] (mM)	$\frac{k_{13}}{k_7}$ [a]	K_{im} [b] (mM)
0.1	0.037 ± 0.016	0.45 ± 0.25	0.039 ± 0.017
0.125	0.037 ± 0.016	0.62 ± 0.33	0.036 ± 0.016
0.167	0.048 ± 0.022	0.51 ± 0.26	0.053 ± 0.023
Weighted mean values	0.039 ± 0.010	0.51 ± 0.16	0.041 ± 0.010

[a] The data for each substrate concentration of three experiments, including that of Figure II.4A, were fitted to equation (10) to give values for K_{iN} and K_{iD}. At $MgIDP^-$ concentrations of 0.1, 0.125 and 0.167 mM, the weighted mean values for K_{iN} were calculated to be 0.0124 ± 0.0005, 0.0093 ± 0.0009 and 0.0123 ± 0.0016 mM, respectively, while those for K_{iD} were determined to be 0.038 ± 0.003, 0.038 ± 0.003 and 0.051 ± 0.007 mM, respectively. These values, together with those for K_{a1}, K_{a2}, k_3/k_7 (Table II.2, column II) and K_A (Table II.6) were used in conjunction with equations (12) and (13) to obtain the true values for K_{im} and k_{13}/k_7 as listed above.

[b] The apparent values for K_{im} at each substrate concentration of three experiments, including that of Figure II.4B, were determined by fitting the data to equation (8). The weighted mean values of apparent K_{im} were then calculated to be 0.041 ± 0.002, 0.038 ± 0.003 and 0.056 ± 0.006 mM at $MgIDP^-$ concentrations of 0.1, 0.125 and 0.167 mM, respectively. These values, together with those for K_{a1}, K_{a2} (Table II.2, column II) and K_A (Table II.6) were substituted into equation (14) to obtain true K_{im} values.

TABLE II.5. : Values for K_{im} and the k_{13}/k_7 ratio as determined from the results of the activation of the reaction by IDP^{3-}[a].

Concentration of $MgIDP^-$ (mM)	K_{im} (mM)	$\frac{k_{13}}{k_7}$
0.1	0.35 ± 0.16	1.0 ± 0.57
0.125	0.34 ± 0.16	1.0 ± 0.54
0.167	0.26 ± 0.11	1.4 ± 0.75
Weighted mean values	0.30 ± 0.08	1.1 ± 0.35

[a]The data for each substrate concentration of two experiments, including that of Figure II.4C, were fitted to equation (10) to give values for K_{iN} and K_{iD}. At $MgIDP^-$ concentrations of 0.1, 0.125 and 0.167 mM, the weighted mean values for K_{iN} were calculated to be 0.100 ± 0.016, 0.106 ± 0.013 and 0.081 ± 0.010 mM, respectively, while those for K_{iD} were determined to be 0.388 ± 0.070, 0.395 ± 0.055 and 0.306 ± 0.036 mM, respectively. These values were used in the calculation of estimates for K_{im} and k_{13}/k_7 as described in Table II.4.

presence and absence of modifiers, respectively, against the reciprocal of the modifier concentration (Figure II.4B). The fact that linear plots are obtained confirms that only one molecule of $MgATP^{2-}$ reacts with the enzyme. The fitting of these data to a rate equation of the same form as equation (8) gave apparent K_{im} values from which the true values were calculated by means of equation (14) and the appropriate kinetic constants. The ratio, k_{13}/k_7, can also be determined by using the vertical intercept values of Figure II.4B, but this calculation was not done because of the complexity of equation (15) and the consequent errors that could arise. From the results presented in Table II.4, it is apparent that both methods for the determination of K_{im} give virtually identical values.

Comparison of the data given in Tables II.4 and II.5 shows that the dissociation constant for the enzyme-IDP complex is about 8 times greater than that for the enzyme-MgATP complex. Further, the value for the former constant is sufficiently high to justify the neglect of the effect of IDP^{3-} when this ionic species is held constant at a concentration of 0.1 mM (cf. Table II.2 and Figure II.2).

Determination of values for K_A. In the above calculations, it was necessary to know the values for K_A, representing the Michaelis constants for the reaction of $MgIDP^-$ with the enzyme-modifier complexes. These were determined by studying the initial velocity of the reaction as a function of the $MgIDP^-$ concentration at different fixed modifier concentrations. The concentration of substrate was varied over the range from 0.2-1.0 mM while $MgATP^{2-}$ and IDP^{3-} were held constant at concentrations ranging from 0.6-3.0 mM and from 1.0-5.0 mM, respectively. The data, which are illustrated in Figure II.5, gave linear double reciprocal plots as required by equation (8) and since, with each modifier, the lines were virtually superimposable then, for all practical purposes, concentrations of $MgATP^{2-}$ and IDP^{3-} at or above 0.6 and 1.0 mM, respectively, may be considered to be saturating. Thus the value obtained for K_A at each modifier concentration can be regarded as a true value and used in the determination of a weighted mean value (Table II.6). The results indicate that there is a significant difference in the magnitude of the K_A values associated with the enzyme-MgATP and enzyme-IDP complexes.

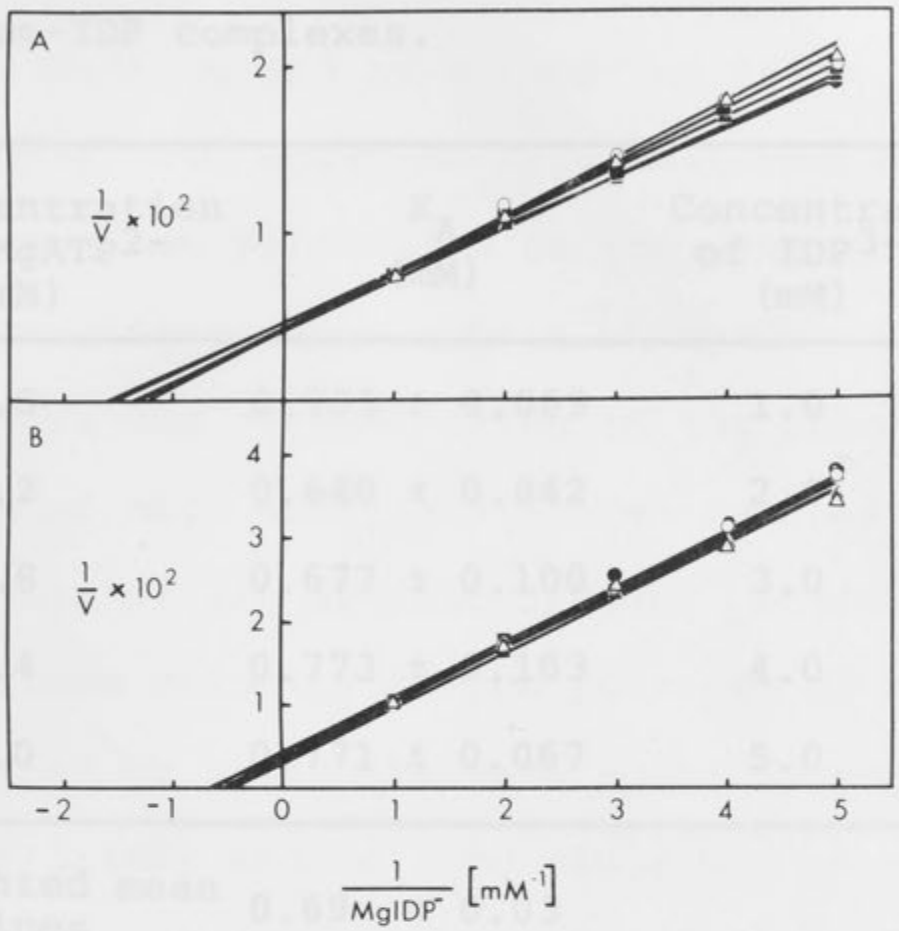


FIGURE II.5. Effects of varying $MgIDP^-$ concentration in the presence of high fixed concentrations of $MgATP^{2-}$, (A) and IDP^{3-}, (B). In (A) the concentrations of $MgATP^{2-}$ were 0.6 (●), 1.2 (▲), 1.8 (■), 2.4 (o) and 3.0 (Δ) mM. In (B) the concentrations of IDP^{3-} were 1.0 (●), 2.0 (▲), 3.0 (■), 4.0 (o) and 5.0 (Δ) mM. v is expressed as mμmoles per min per μg of protein.

TABLE II.6. : Values of the kinetic constants $(K_A)^a$ for the reaction of $MgIDP^-$ with the enzyme-MgATP and enzyme-IDP complexes.

Concentration of $MgATP^{2-}$ (mM)	K_A (mM)	Concentration of IDP^{3-} (mM)	K_A (mM)
0.6	0.733 ± 0.069	1.0	1.46 ± 0.28
1.2	0.640 ± 0.042	2.0	1.71 ± 0.14
1.8	0.677 ± 0.100	3.0	1.92 ± 0.22
2.4	0.773 ± 0.103	4.0	2.20 ± 0.34
3.0	0.771 ± 0.067	5.0	1.58 ± 0.13
Weighted mean values	0.69 ± 0.03		1.69 ± 0.08

[a]Values for K_A were obtained by fitting the data of Figure II.5 to equation (8).

Determination of maximum velocities in the presence and absence of modifiers. As a more direct demonstration of the effect of modifiers on the maximum velocity of the reaction and as a check on the k_{13}/k_7 value calculated from equation (12), determinations were made of the maximum velocities in the presence and absence of saturating concentrations of $MgATP^{2-}$ and IDP^{3-}. To facilitate the determination in the absence of modifier, a range of high substrate concentrations was used so that plots of 1/v against $1/MgIDP^-$ would be linear. The maximum velocity in the presence of modifier was determined by using the same range of high substrate concentrations and holding the ratio of substrate: modifier constant. The initial velocity data illustrated in Figure II.6 in the form of double reciprocal plots were corrected, when necessary, for the inhibition by free Mg^{2+}. But it should be noted that when IDP^{3-} was varied in constant ratio with the substrate, no correction was necessary because the relatively high concentrations of free IDP^{3-} kept free Mg^{2+} at concentrations well below those at which inhibition occurs. The results indicate quite clearly that while both modifiers increase the reaction velocity when substrate is present at lower concentrations, their

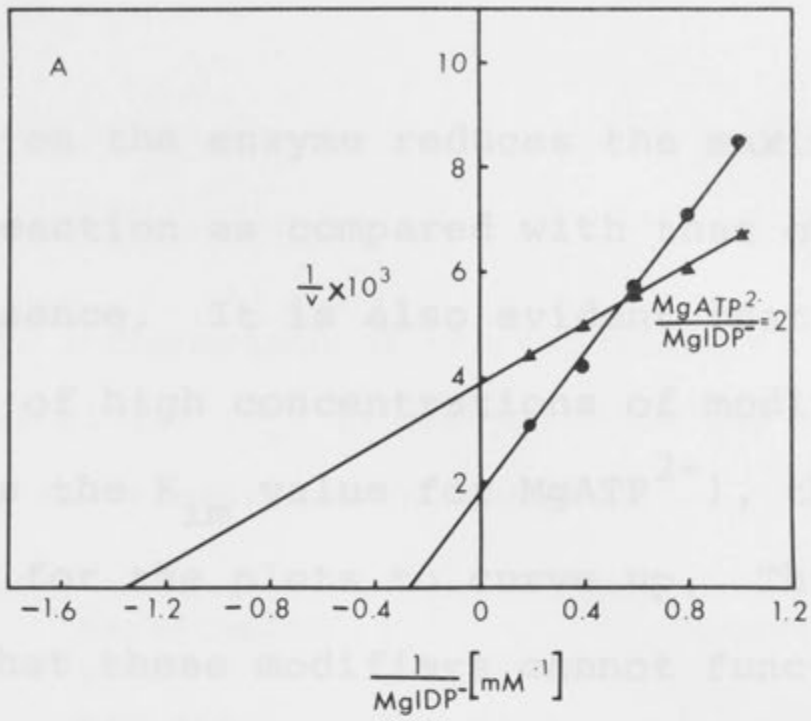


FIGURE II.6. Effects of the concentration of $MgIDP^-$ on the initial velocity of the reaction in the presence and absence of $MgATP^{2-}$ (A) and higher concentrations of free IDP^{3-} (B). For the control experiments (●), free IDP^{3-} was held constant at 0.1 mM while the effects of $MgATP^{2-}$ and higher concentrations of free IDP^{3-} were determined by varying their concentrations in constant ratio with respect to that of the substrate (▲). Before fitting to equation (8), all data sets were corrected for inhibition by free Mg^{2+} as described in Table II.2 using the constants given in Table II.3. v is expressed as mμmoles per min per μg of protein.

presence on the enzyme reduces the maximum velocity of the reaction as compared with that obtained in their absence. It is also evident that in the presence of high concentrations of modifier (up to 250 times the K_{im} value for $MgATP^{2-}$), there is no tendency for the plots to curve up. Thus it would appear that these modifiers cannot function as inhibitors of the reaction by combining with both sites at which $MgIDP^{-}$ can react so as to form dead-end modifier-enzyme-modifier complexes.

Analysis of the data of Figure II.6 gave the maximum velocity values which are listed in Table II.7 and these were used to calculate the ratios of the determined maximum velocities in the presence and absence of saturating concentrations of the modifiers. Such values represent only apparent k_{13}/k_7 ratios and hence cannot be compared directly with the corresponding true values given in Tables II.4 and II.5. The two sets of values are of a similar magnitude, but on the basis of the proposed mechanism this would not necessarily be expected to be so because of the differences inherent in the two types of experiment described in Figures II.4 and II.6. Analysis of the data of Figure II.4, in effect, involves the separate

TABLE II.7. : Comparison of the experimental and calculated maximum velocities (V_m) in the presence and absence of saturating concentrations of modifiers[a].

Modifier	Experimental V_m[b]		Apparent[d] $\frac{k_{13}}{k_7}$ Ratio	True[e] $\frac{k_{13}}{k_7}$ Ratio
	No Modifier[c]	Plus Modifier		
$MgATP^{2-}$	558 ± 20	260 ± 1	0.47 ± 0.02	0.46 ± 0.22
IDP^{3-}	301 ± 13	146 ± 15	0.49 ± 0.05	0.42 ± 0.22

[a]Each value is the weighted mean of values obtained from two experiments, including those shown in Figure II.6.

[b]V_m is expressed as mμmoles per min per μg of protein.

[c]The V_m values in the absence of modifier are not similar because of the difference in the specific activity of the enzyme preparations used for the two experiments.

[d]Ratio of V_m in the presence and absence of saturating concentrations of the modifiers.

[e]These values were calculated by using equation (18) and the apparent k_{13}/k_7 ratio, as well as the values for K_{a1}, K_{a2} (Table II.2, column II), K_{im} (Tables II.4 and II.5) and K_A (Table II.6).

extrapolation to infinite concentrations of both substrate and modifier (cf. equations 10 and 11) and this procedure is similar to that used for determining the maximum velocity of any reaction involving two reactants. On the other hand, when substrate and modifier are varied in constant ratio (Figure II.6), the vertical intercept of a double reciprocal plot represents the situation where both reactants are present simultaneously at infinite concentrations so that two enzyme complexes, substrate-enzyme-modifier and substrate-enzyme-substrate, are present. The value of the intercept would depend not only on the proportion of total enzyme in each form, determined by the values of the kinetic constants for the reaction of substrate and modifier with the enzyme, but also on the rate at which each complex gave rise to products. The relationship between the maximum velocities of the reaction in the presence and absence of a saturating concentration of modifier is given by equation (18) which was used, together with the data of Figure II.6 and the values for the appropriate kinetic constants, to determine the true k_{13}/k_7 ratios. The results (Table II.7) indicate that the value with $MgATP^{2-}$ as modifier is in good agreement with that given in Table

II.4, while the value with IDP^{3-} as modifier is less than that recorded in Table II.5. However, when the standard errors of the latter two values are taken into account, the difference is not large. Since the determination of the k_{13}/k_7 ratio by the approach outlined in Figure II.6 can be regarded as being more direct than that illustrated in Figure II.4, the value of 0.42 ± 0.22 with IDP^{3-} as modifier, should be considered to be the more correct of the two reported values. Consequently, the conclusion can be reached that the MgIDP-enzyme-MgATP and MgIDP-enzyme-IDP complexes (AEM) break down to yield products at slower rates than the MgIDP-enzyme-MgIDP complex (AEA), but at faster rates than the enzyme-MgIDP complex (EA). Irrespective of which nucleotide is the modifier, the relative rates of breakdown of the three complexes are approximately : AEA, 100; MEA, 45; EA, 8.

Effect of saturating concentrations of $MgATP^{2-}$ and IDP^{3-} on the velocity of the reaction. While the above experiments provided no evidence for the formation of dead-end enzyme-modifier complexes as a result of the modifier occupying both substrate sites, it was possible that such a complex might be formed in the simultaneous presence of high concentrations of both

modifiers and a low substrate concentration. However, Figure II.7 shows that no inhibition occurred when both $MgATP^{2-}$ and free IDP^{3-}, in a constant ratio of 1, were increased simultaneously over concentration ranges which were high compared with their dissociation constants (Tables II.4 and II.5) and $MgIDP^{-}$ was kept at a concentration of 0.2 mM, which is below its K_m value (Table II.2). It appears, therefore, that modifiers can react at only one of the two sites at which substrate can combine.

Kinetics of the reaction when the concentrations of IDP^{3-} and $MgIDP^{-}$ are varied in constant ratio. When the ratio of IDP^{3-}:$MgIDP^{-}$ is held constant at a value of 0.5, the concentration of free Mg^{2+} does not increase to inhibitory levels as the concentration of $MgIDP^{-}$ is increased. Rather, it remains constant at a concentration of 0.5 mM which can be considered to have only a negligible effect on the reaction. Thus the study of the initial velocities of the reaction as a function of the concentration of $MgIDP^{-}$, while maintaining the IDP^{3-}:$MgIDP^{-}$ ratio constant, offers a potential means of avoiding the complications arising from the inhibition by free Mg^{2+}. In addition, it provides an alternative method of obtaining values for

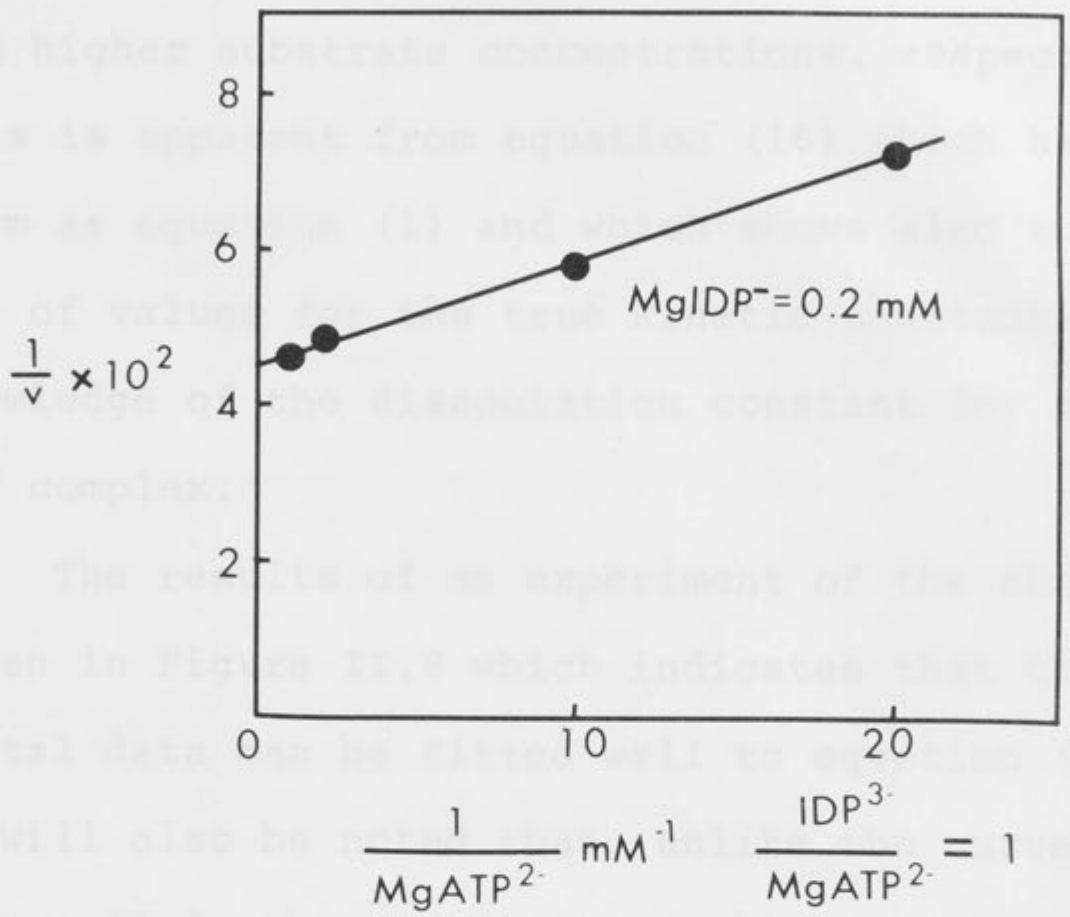


FIGURE II.7. Effect of increasing modifier concentrations on a single fixed concentration (0.2 mM) of $MgIDP^-$. $MgATP^-$ and IDP^{3-} were maintained at a constant ratio of 1.0. The line has been fitted by eye. v is expressed in arbitrary units.

the kinetic constants of the reaction. However, such an approach is not straightforward because of both the activating and inhibitory effects of free IDP^{3-} at lower and higher substrate concentrations, respectively. This is apparent from equation (16) which has the same form as equation (1) and which shows also that calculation of values for the true kinetic constants requires a knowledge of the dissociation constant for the enzyme-IDP complex.

The results of an experiment of the above type are given in Figure II.8 which indicates that the experimental data can be fitted well to equation (16). But it will also be noted that, unlike the curves of Figure II.2, the asymptote of this curve is not well defined and hence large standard errors are associated with the values for the kinetic constants (Table II.8). It would be anticipated that more precise values could be obtained by further reducing the substrate concentration, but it was not possible to determine initial velocities at concentrations of $MgIDP^{-}$ less than 0.1 mM. By way of comparison, the values for the kinetic constants as determined by allowing for the effects of free Mg^{2+} on one hand and for free IDP^{3-} on the other, are listed in Table II.8. The two sets are

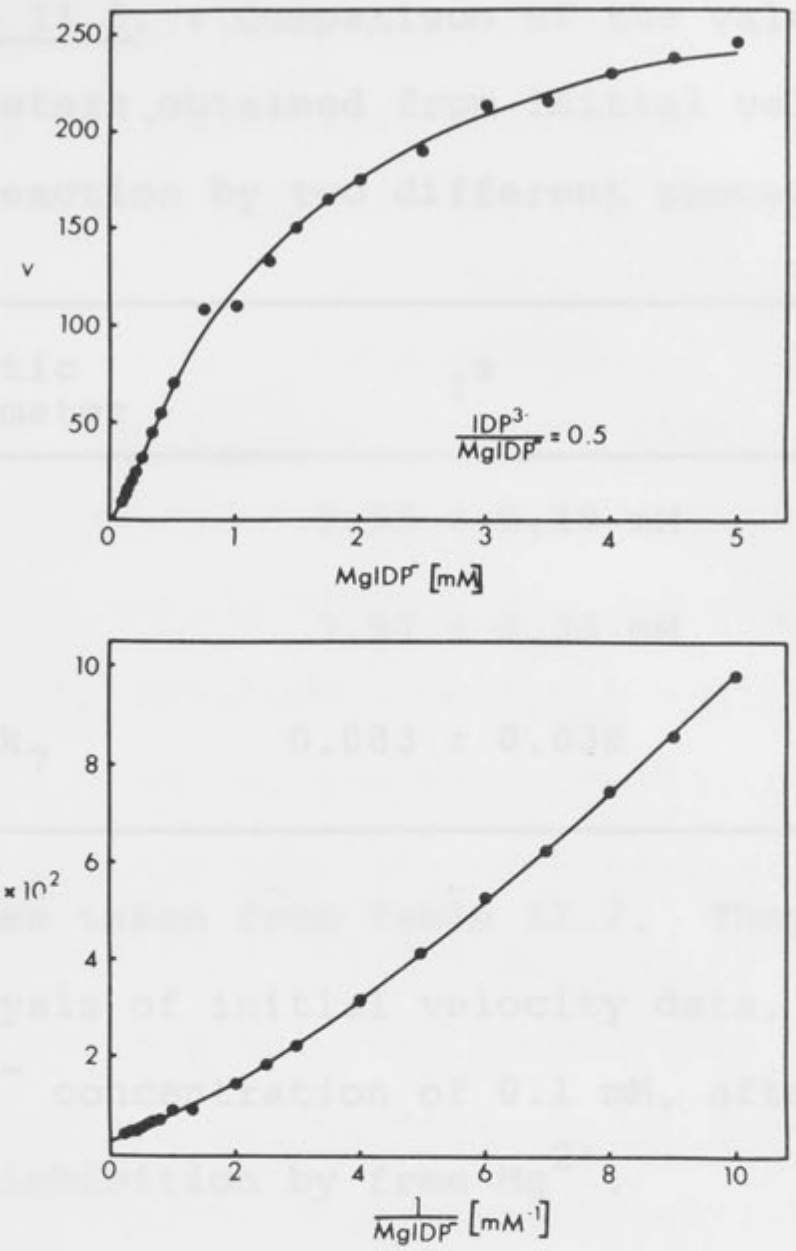


FIGURE II.8. Effect of substrate concentration on the initial velocity of the reaction under conditions where the ratio of $MgIDP^-$:free IDP^{3-} was held constant. The lines represent the theoretical curves which were drawn by using the constants obtained from fitting the data to equation (1). v is expressed as mμmoles per min per μg of protein.

TABLE II.8. : Comparison of the values for the kinetic parameters obtained from initial velocity studies of the reaction by two different procedures.

Kinetic Parameter	I[a]	II[b]
K_{a1}	0.55 ± 0.19 mM	0.31 ± 0.19 mM
K_{a2}	3.97 ± 0.33 mM	1.32 ± 0.54 mM
k_3/k_7	0.083 ± 0.036	0.035 ± 0.040

[a]Values taken from Table II.2. They were obtained by analysis of initial velocity data, at a fixed free IDP^{3-} concentration of 0.1 mM, after correction for the inhibition by free Mg^{2+}.

[b]The data of Figure II.8 were fitted to equation (17) to give values for b', c' and d' of 1.43 ± 0.17, 0.289 ± 0.119 and 0.044 ± 0.042 mM, respectively. The values for c', d', X, K_A (Table II.6) and K_{im} (Table II.5) were substituted into equations (20) and (21) to give values for K_{a1} and K_{a2}. These were then used, together with values for d', k_{13}/k_7 (Table II.7), K_A, K_{im} and X to determine values for k_3/k_7 from equation (19).

similar, but the high standard errors of one set does not permit any conclusion to be drawn about the accuracy of the kinetic constants obtained by the correction, as applied, for the inhibition by free Mg^{2+}.

Discussion

The kinetic data obtained from a study of the reaction catalyzed by nucleoside diphosphatase are consistent both qualitatively and quantitatively with the ordered reaction hypothesis outlined in the Theory section. That is, the experimental results are in accord with the idea that the native enzyme reacts with one molecule of substrate to form an enzyme-substrate complex which gives rise to products and which reacts further with a second molecule of substrate to yield a substrate-enzyme-substrate complex that also breaks down to give products. This reaction sequence is manifested in the shape of a double reciprocal plot of initial velocity as a function of substrate concentration that has the form of a non-rectangular hyperbola which approximates to two straight lines of differing slopes at high and low substrate concentrations. It is this shape that excludes the possibility that the first molecule of substrate to react functions as an

essential modifier, rather than as a substrate. If this were so, a parabolic double reciprocal plot would be obtained (cf. Worcel, Goldman and Cleland, 1965).

The conclusion that two molecules of substrate are involved in the reaction does not preclude the possibility that this particular enzyme is made up of more than two subunits and that more than two substrate molecules react per mole of enzyme. If, for instance, the enzyme contained four subunits which were arranged as two equivalent pairs, then kinetic techniques would give evidence for the reaction of only two molecules of substrate. Thermodynamic experiments would be required to determine the number of moles of substrate combining per mole of enzyme. It should also be mentioned that kinetic techniques might only give a minimum value for the number of substrate molecules reacting. Determination of the total number requires that the magnitude of the combination constants be such as to make kinetically significant the steady-state concentration of each enzyme-substrate complex.

The results are also in accord with the idea that the modifiers, $MgATP^{2-}$ and IDP^{3-}, which are structurally related to $MgIDP^{-}$, can combine in a mutually exclusive manner at one substrate site on the enzyme to form an

enzyme-modifier complex with which only one molecule of substrate can react. Such a finding is in agreement with the conclusion that two molecules of substrate are capable of reacting with the native enzyme. In combining with the enzyme, both modifiers apparently cause a similar conformational change which prevents the reaction of a second molecule of modifier and the formation of dead-end modifier-enzyme-modifier complexes. The values of the Michaelis constants (K_A) associated with the reaction of substrate with the enzyme-modifier complexes are either similar to or greater than the value of the combination constant (K_{al}) for the reaction of substrate with free enzyme, while the rates of product formation from the substrate-enzyme-modifier complexes are greater than the rate of product release from the enzyme substrate complex. On the basis of the applied steady-state treatment for an ordered reaction, such findings imply that the dissociation constants for the substrate-enzyme-modifier complexes are less than the dissociation constant for the enzyme-substrate complex. Hence it would follow that the effect of the modifiers of the nucleoside diphosphatase reaction is not only to increase the binding of substrate, but also to increase the rate of product formation. If,

however, K_A and K_{al} represented dissociation constants, then it would be concluded that the activating effect of the modifiers is related to the formation of a substrate-enzyme-modifier complex which yields product at a faster rate than the enzyme-substrate complex. Thus the effect of a modifier in reducing an apparent K_m value for a substrate cannot necessarily be interpreted as indicating an increase in the binding of that substrate, although this conclusion has been reached frequently. Although the presence of modifier on the enzyme results in a greater rate of product formation at low substrate concentrations, this rate is less than that obtained when substrate is saturating and all enzyme is in the form of a substrate-enzyme-substrate complex. However, it is unlikely that the concentration of $MgIDP^-$ required for saturation is ever attained under in vivo conditions.

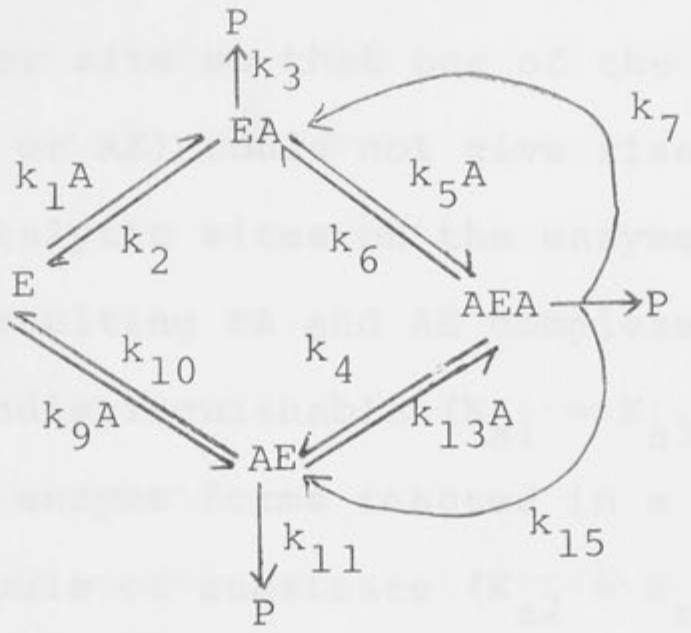
When referred to in a general sense, IDP^{3-} and $MgATP^{2-}$ have been considered as modifiers. The reason for this is that the present results show quite clearly that these nucleotide species function as activators only under conditions where the substrate concentration is low compared with its K_m value, while at higher substrate concentrations, they act as inhibitors of the

reaction. As a consequence of these dual effects, there will be a substrate concentration at which a particular concentration of modifier will cause neither activation nor inhibition. In connection with the characterization of a modifier, it is of importance to carry out experiments over wide ranges of both substrate and modifier concentrations. The present results also raise the question of the meaning of the term, allosteric activator, and draw attention to the possibility that a more rigid classification of modifiers, based on their differential ability to influence the binding of substrate and the maximum velocity of the reaction, will be necessary.

Because the experimental results are consistent with the theoretical postulates, it cannot be concluded that the reaction does occur via the proposed mechanism. Indeed, a number of mechanisms can give rise to an initial velocity equation of the same form as that represented by equation (1) and some of these are discussed below.

If the native enzyme possessed two non-equivalent active sites at which substrate could add in a random manner and these steps were rapid compared with the rate of breakdown of all three enzyme-substrate

complexes, then the initial velocity equation for the reaction mechanism (Scheme II.1)



Scheme II.1

would become

$$v = \frac{V\left[A^2 + \left(\frac{k_3}{k_7 + k_{15}} K_{a2} + \frac{k_{11}}{k_7 + k_{15}} K_{a4}\right) A\right]}{A^2 + \left(K_{a2} + K_{a4}\right)A + K_{a1}K_{a2}} \qquad (23)$$

where $K_{a1} = \frac{k_2}{k_1}$, $K_{a2} = \frac{k_6}{k_5}$, $K_{a4} = \frac{k_{14}}{k_{13}}$, and $V = \left(k_7 + k_{15}\right)e_t$. Further, $K_{a1}K_{a2}$ would equal $K_{a3}K_{a4}$ where $K_{a3} = \frac{k_{10}}{k_9}$. Apart from being unable to distinguish, by means of initial velocity studies, this reaction mechanism from that proposed in the Theory section, it would not be possible to determine values for the individual kinetic constants associated with the reaction of substrate with the enzyme or for the

ratios of rate constants. Similar remarks would apply if one of the sites at which substrate could combine were a modifier site so that one of the enzyme-substrate complexes (EA or AE) could not give rise to products. If the two catalytic sites on the enzyme were equivalent so that the resulting EA and AE complexes were kinetically indistinguishable ($K_{a1} = K_{a3}$ and $k_3 = k_{11}$) and these two enzyme forms reacted in a similar way with a second molecule of substrate ($K_{a2} = K_{a4}$) to give an AEA complex whose sites were equivalent with respect to product formation ($k_7 = k_{15}$), then equation (23) would simplify to

$$v = \frac{V\left(A^2 + \frac{k_3}{k_7} K_{a2}A\right)}{A^2 + 2K_{a2}A + K_{a1}K_{a2}} \qquad (24)$$

where $V = 2k_7e_t$. For this condition, the determined value for K_{a2} would be twice the true value and as a consequence of this, the calculated values for K_{a1} and the k_3/k_7 ratio would be half the true values. Furthermore, if the two sites on the enzyme were not only equivalent but the two substrate molecules reacted independently, then in addition to the relationships given above, one would have $K_{a1} = K_{a2}$ and $k_3 = k_7$. Because of the presence of a statistical factor in the

denominator of equation (24), it would reduce to $v = \frac{VA}{K_{a2} + A}$ and a linear double reciprocal plot would be obtained. It is possible that, with some allosteric enzymes, modifiers could exert their effects by combining at a specific site on the enzyme and altering the properties of the catalytic sites in this manner. However, such a postulate is not tenable for the nucleoside diphosphatase reaction. As the maximum velocity of the reaction is not affected by the addition of high, finite concentrations of modifiers, then on the basis of the above hypothesis, it would have to be concluded that the enzyme complex involving two molecules of substrate and one molecule of modifier breaks down at the same rate as that involving only two molecules of substrate. But such a conclusion would not be consistent with the finding that saturating concentrations of $MgATP^{2-}$ and IDP^{3-} reduce the maximum velocity of the reaction (Figure II.6). It should also be noted that the initial velocity equation (1) would be obtained if the ordered mechanism proposed in the Theory section were of the rapid equilibrium, random type so that the rate of breakdown of both the EA and AEA complexes were slow compared with all other steps of the reaction sequence. Under

these circumstances, the combination constants, K_{a1} and K_{a2}, would represent dissociation constants.

If the rate of product formation for the mechanism shown in Scheme II.1 were not rate limiting so that the reaction conformed to steady-state kinetics, then both the numerator and denominator of the initial velocity equation would contain cubic terms in A. The fact that the experimental data can be fitted to the simpler form of initial velocity equation could be taken to indicate that this steady-state mechanism can be eliminated from consideration. However, it should be borne in mind that it may well be difficult by curve-fitting techniques to distinguish between initial velocity equations such as equation (3) and those containing cubic and higher power terms in substrate concentration, especially when double reciprocal plots of velocity as a function of substrate concentration exhibit only a small curvature. Parenthetically, it might be mentioned that if an enzyme molecule was composed of four catalytic units, and four substrate molecules reacted interdependently, then the initial velocity equation in reciprocal form would contain up to fifth and fourth power substrate concentration terms in the numerator and denominator,

respectively when steady-state conditions are assumed. Alternatively, when equilibrium conditions apply, the equation would have up to fourth and third power terms in the numerator and denominator, respectively. It should also be emphasized that there is no justification for fitting data to more complex velocity equations, which contain an increased number of constants that thereby facilitate the fitting, unless it has been shown that such data cannot be fitted to simpler equations.

A simplification of the steady-state initial velocity equation for the random type mechanism (Scheme II.1) will occur when it is assumed that the two sites on the enzyme are equivalent and that they react with substrate to give EA and AE complexes that are also equivalent. From these assumptions it follows that $k_1 = k_9$, $k_2 = k_{10}$, $k_5 = k_{13}$, $k_6 = k_{14}$, $k_3 = k_{11}$ and $k_7 = k_{15}$ and the initial velocity equation becomes

$$v = \frac{V\left[A^2 + \frac{k_3}{k_7} K_{a2}A\right]}{A^2 + 2K_{a2}A + K_{a1}K_{a2}} \qquad (25)$$

where $V = 2k_7e_t$, $K_{a1} = \frac{k_2 + k_3}{k_1}$ and $K_{a2} = \frac{k_6 + k_7}{k_5}$.

This equation is identical with equation (24) and reduces to a linear form when $k_3 = k_7$ and $K_{a1} = K_{a2}$. Thus under special conditions, initial velocity equations for a steady-state mechanism involving the random addition of two molecules of substrate to an enzyme can have the same form as those obtained when an ordered sequence of combination occurs (equation 1) or when rapid equilibrium conditions apply (equation 24).

Rapid equilibrium mechanisms which allow for the reaction of modifier only after the addition of one molecule of substrate can be eliminated from consideration since in these cases the maximum velocity of the reaction, in the presence of a saturating concentration of modifier, would be independent of the substrate concentration over the ranges of substrate concentration used in initial velocity determinations. In the experiments which were designed to determine the values for K_A and for which the modifiers were present at what can be considered as being saturating concentrations, it was found that the initial velocity did vary as a function of the substrate concentration. Further, it is unnecessary to consider any steady-state mechanism which allows for the reaction of modifier with both the enzyme

and enzyme-substrate complexes as the initial velocity equation would contain squared terms in modifier concentration and the initial velocity would be a complex, rather than a hyperbolic function of the modifier concentration (Figure II.4). However, such a mechanism could not be excluded if the reaction conforms to rapid equilibrium kinetics for then the initial velocity equation would be of the same form as equation (7).

From the above discussion, it becomes apparent that initial velocity studies have severe limitations with respect to the elucidation of the mechanisms of allosteric enzyme reactions, even when the ideas of classical kinetic theory are applicable. But, of course, these same limitations also apply in relation to the investigations on enzymes which exhibit Michaelis-Menten kinetics (Cleland, 1963a). It will be essential, therefore, to carry out product inhibition and isotope exchange studies to gain further information about the mechanism of the reaction catalyzed by nucleoside diphosphatase. In addition, the elucidation of the reaction mechanism would be facilitated by performing binding studies, similar to those reported by Changeux, Gerhart and Schachman (1968), with

modifiers and compounds clearly shown to function as linear competitive inhibitors of nucleoside diphosphatase because of their combination at the same site(s) as substrate. But this type of experiment has been precluded by the limited amounts of enzyme available (see Chapter I).

No consideration has been given here to the interpretation of the results on the basis of other hypotheses that have been proposed and which are not readily susceptible to experimental test (Monod et al., 1965; Koshland et al., 1966; Kirtley and Koshland, 1967; Nichol et al., 1967). However, it is realized that isomerization and monomer-polymer interactions of the native enzyme could also be involved in the reaction sequence. But the aim of these studies has been achieved in that it has been demonstrated that the results for one allosteric enzyme reaction can be accounted for in terms of a steady-state kinetic theory. This finding has particular significance in view of the widespread neglect of the possibility that allosteric effects might well be interpretable in terms of classical theories of enzyme action. At the same time, it must be emphasized that the agreement between the experimental results and those predicted on the basis of the

proposed hypothesis does not constitute proof of the correctness of the hypothesis.

Summary

A highly purified preparation of nucleoside diphosphatase has been used to study the kinetics of the reaction at pH 8.5 under conditions where Mg-inosine diphosphate ($MgIDP^-$) was the variable substrate and the concentration of IDP^{3-} was controlled. Double reciprocal plots of initial velocity as a function of the $MgIDP^-$ concentration at a relatively low, fixed concentration of IDP^{3-} yielded concave-up non-rectangular hyperbolas. These data were shown to give good fits to a steady-state initial velocity equation which was derived on the assumptions that two molecules of substrate (A) reacted with the enzyme (E) in an inter-dependent ordered manner ($E \rightleftharpoons EA \rightleftharpoons AEA$) and that both the EA and AEA complexes gave rise to products. Sufficiently high concentrations of the allosteric modifiers, free IDP^{3-} and Mg-adenosine triphosphate ($MgATP^{2-}$) caused the above type of double reciprocal plot to become linear which indicates that, under these conditions, only one molecule of substrate undergoes reaction with the enzyme. From investigations of

initial velocity as a function of the concentrations of $MgATP^{2-}$ and free IDP^{3-} it was concluded that only one molecule of each of the modifiers can react with the enzyme. The maximum velocity of the reaction with $MgIDP^{-}$ at a saturating concentration was reduced in the presence of saturating concentrations of both $MgATP^{2-}$ and free IDP^{3-}. Values have been obtained for the kinetic constants associated with the reactants, as well as for the maximum velocity of the reaction under different conditions. The various hypotheses which are consistent with the experimental results have been discussed.

CHAPTER III

THE ACTIVATION OF NUCLEOSIDE DIPHOSPHATASE BY VARIOUS MAGNESIUM-NUCLEOSIDE TRIPHOSPHATE COMPLEXES

CHAPTER III

THE ACTIVATION OF NUCLEOSIDE DIPHOSPHATASE BY VARIOUS MAGNESIUM-NUCLEOSIDE TRIPHOSPHATE COMPLEXES

Introduction

The results reported in Chapters I and II indicated that the magnesium-nucleoside triphosphates, $MgATP^{2-}$, $MgCTP^{2-}$, $MgUTP^{2-}$, $MgITP^{2-}$ and $MgGTP^{2-}$, as well as the free nucleotide, IDP^{3-}, act as activators of nucleoside diphosphatase when $MgIDP^{-}$ is used as the substrate, and that both $MgATP^{2-}$ and IDP^{3-} are not changed in the presence of enzyme. Further, the results obtained with two of the modifiers, $MgATP^{2-}$ and IDP^{3-}, were in accord with the proposal that they function by combining at one of the two substrate sites on the enzyme and thereby bring about an alteration of its catalytic properties. The effects of these two modifiers were similar in that saturating concentrations of either had the effect of increasing the reaction rate at lower, while decreasing the rate at higher substrate concentrations. Furthermore, quantitative analysis of the results of kinetic studies with $MgATP^{2-}$ and IDP^{3-} gave values for the dissociation constants of the enzyme-modifier complexes as well as the relative rates of breakdown of the AEA

and MEA complexes according to the proposed theory.

The techniques which were developed for studying the effects of IDP^{3-} and $MgATP^{2-}$ mentioned above may be applied equally well to the determination of the effects on the reaction of other allosteric modifiers. The results of such investigations are of interest, for they enable a comparison to be made of the affinity with which different modifiers combine with the enzyme, as well as their effects on the maximum velocity of the reaction. Results of such studies which are reported in this chapter, are in agreement with the idea that the various modifiers can form enzyme-modifier complexes which differ in their kinetic properties.

Experimental

Materials and methods. The materials and methods used for this study were as described previously (Chapter I) with the following additions. All nucleoside and deoxynucleoside triphosphates were products of P-L Biochemicals. The deoxynucleotides were used without further purification, however, the nucleoside triphosphates, ITP, UTP, GTP and CTP, had been stored as the crystalline sodium salts at -10°C for about two years and hence contained 6-10% of the corresponding diphosphates.

It was not necessary to purify ITP, as allowance could be made for the contaminating IDP when calculating the substrate ($MgIDP^-$) concentrations to be used. Further, purification of CTP was unnecessary because $MgCDP^-$ is a poor substrate and the amount present would not interfere with the reaction. It was necessary to purify UTP and GTP, since $MgUDP^-$ and $MgGTP^-$ are good substrates and hence could cause erroneous results if present. The nucleotides were purified according to a modification of the method of Hurlbert (1957). About 200 mg of the nucleotide sample was adsorbed onto a small (6 x 1.5 cm) column of Dowex-1 (200-400 mesh) formate and the nucleotides eluted by use of linear gradients of 0.4-1.0 and 0.2-0.8 N ammonium formate in 4 N formic acid. The total volume of eluant was 200 ml. After elution from the column, the nucleoside triphosphates were adsorbed onto a pad of charcoal in a small buchner funnel, and eluted with 1:1 water:ethanol. The eluant from the last step was made 85% with respect to ethanol and the nucleotide was precipitated by the addition of a 50% molar excess of a 3% (w/v) $BaBr_2$ solution. The precipitate was washed with absolute ethanol, followed by a second wash with anhydrous ether, and dried. The sodium salt was obtained by mixing a

slurry of the barium-nucleotide into the top of a small (1.8 x 9 cm) column of Zeo Carb 225 (Na^+ form) and washing through with H_2O. After this treatment, UTP and GTP contained approximately 1% of the corresponding nucleoside diphosphates as determined by an assay method which couples the pyruvate kinase and lactic dehydrogenase reactions (Morrison and James, 1965).

MgNTP's were considered inert as substrates for the reaction, and this point was borne out by observations with $MgITP^{2-}$, that stoichiometric amounts of Pi and IMP were released from $MgIDP^-$ both in the presence and absence of the $MgITP^{2-}$. This observation is in accord with the previously shown results (Chapter I) that $MgATP^{2-}$ is unchanged by nucleoside diphosphatase both in the presence and absence of the substrate.

Analysis of data. Experimental data were analyzed by the computer programs of Cleland (1963d). Data which gave linear double reciprocal plots were fitted to equation (1) :

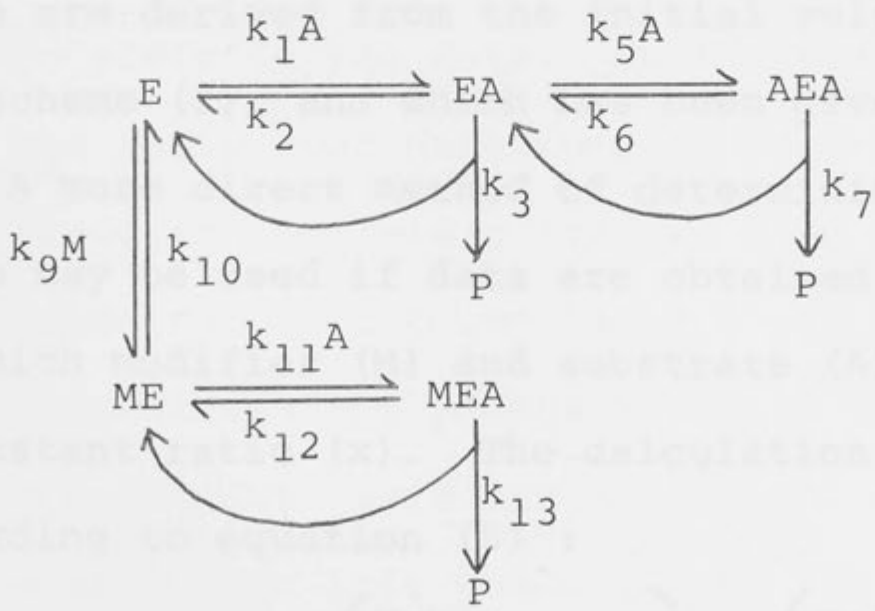
$$v = \frac{VA}{K_A + A} \qquad (1)$$

to determine values for K_A and V. Data obtained from

a study of the effect of magnesium nucleoside triphosphates (M) on the initial velocity of the reaction gave rectangular hyperbolas that did not pass through the origin in plots of initial velocity against the concentration of M and were fitted to equation (2) :

$$v = \frac{V\left(1 + \frac{M}{K_{iN}}\right)}{\left(1 + \frac{M}{K_{iD}}\right)} \qquad (2)$$

where V represents the initial velocity in the absence of modifier. The values so obtained for K_{iN} and K_{iD} were used in calculations which gave values for the dissociation constants of the enzyme-modifier complexes and the relative rates of breakdown of the MEA and AEA complexes in the proposed reaction scheme (I).



(I)

Where the dissociation constant for the enzyme modifier complex, $K_{im} = k_{10}/k_9$, and the Michaelis constant for the reaction of substrate with the ME complex, $K_A = \frac{k_{12} + k_{13}}{k_{11}}$. The relative rate of breakdown of the MEA and AEA complexes is given by k_{13}/k_7.

Determination of constants. The rate and kinetic constants described above may be calculated from the relationships (3) and (4) :

$$K_{im} = \frac{K_{iD}K_{a1}K_{a2}\left(1 + \frac{A}{K_A}\right)}{A^2 + K_{a2}A + K_{a1}K_{a2}} \qquad (3)$$

$$k_{13}/k_7 = \frac{K_A K_{im}\left(A + \left(\frac{k_3}{k_7}\right)K_{a2}\right)}{K_{iN}K_{a1}K_{a2}} \qquad (4)$$

which are derived from the initial velocity equation for scheme (I), and which has been given in Chapter II.

A more direct method of determining the k_{13}/k_7 ratio may be used if data are obtained from experiments in which modifier (M) and substrate (A) are varied in a constant ratio (x). The calculation can be made according to equation (5) :

$$k_{13}/k_7 = \frac{V'}{V}\left(\frac{K_A K_{im}}{K_{a1}K_{a2}x} + 1\right) - \left(\frac{K_A K_{im}}{K_{a1}K_{a2}x}\right) \qquad (5)$$

where x = M/A, and V' and V are the maximum velocities in the presence and absence of modifier, respectively.

Weighted mean values and standard errors of the kinetic constants were calculated according to the equations given in Chapter II (p.104).

Results

Kinetics of the reaction at high fixed concentrations of different modifiers. The effects of varying substrate concentrations in the presence of high fixed concentrations of the magnesium-nucleoside triphosphate (MgNTP) modifiers are shown in Figure III.1. Plots of 1/v against $1/MgIDP^-$ appear as straight lines, as is required by scheme (I) when M is present at high concentrations. Further, increasing the levels of M at concentrations above 0.6 or 1.0 mM does not cause any change in the kinetic plots of the data. Such a result is consistent with the above concentrations of activator being sufficient to completely convert E to the ME complex in which case only one molecule of A can react. Under these conditions the points at which the lines intersect the $1/MgIDP^-$ axis are equivalent to $-1/K_A$, and it may be seen that this value is not the same for all of the modifiers used.

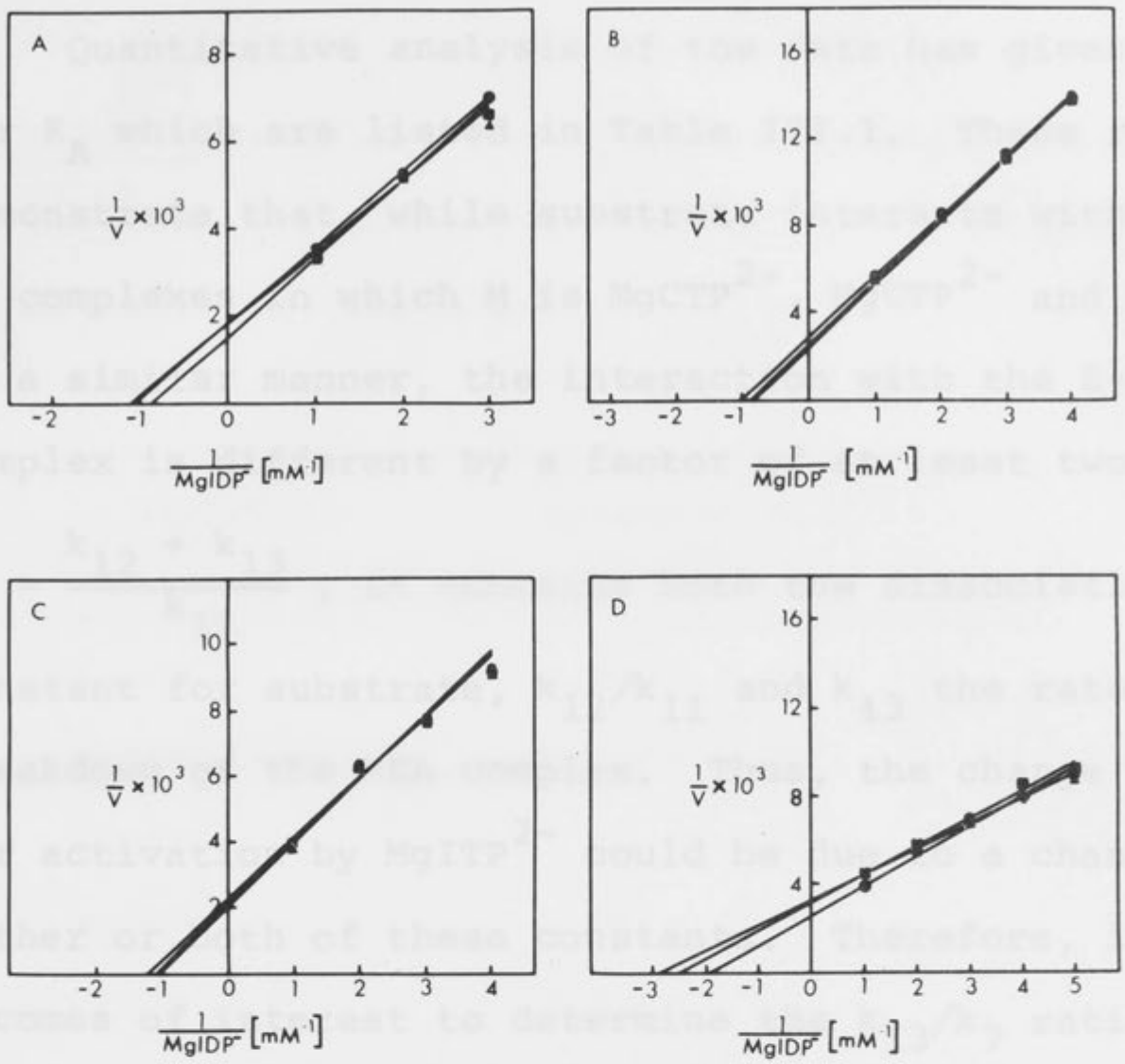


FIGURE III.1. : The effect of varying substrate concentrations at high fixed concentrations of : (A) $MgGTP^{2-}$; 0.62(●), 1.26(▲) and 1.89(■) mM. (B) $MgCTP^{2-}$, (C) $MgUTP^{2-}$ and (D) $MgITP^{2-}$; 1(●), 2(▲) and 3(■) mM. The lines were drawn using the kinetic constants obtained from fitting the data to equation (1). Initial velocity is expressed as mμmoles of Pi/min/μg of protein in (D) and as mμmoles of IMP/min/μg of protein in (A), (B) and (C).

Quantitative analysis of the data has given values for K_A which are listed in Table III.1. These results demonstrate that, while substrate interacts with the EM complexes in which M is $MgCTP^{2-}$, $MgUTP^{2-}$ and $MgGTP^{2-}$ in a similar manner, the interaction with the E-$MgITP^{2-}$ complex is different by a factor of at least two. As $K_A = \frac{k_{12} + k_{13}}{k_{11}}$, it contains both the dissociation constant for substrate, k_{12}/k_{11} and k_{13} the rate of breakdown of the MEA complex. Thus, the change in K_A for activation by $MgITP^{2-}$ could be due to a change in either or both of these constants. Therefore, it becomes of interest to determine the k_{13}/k_7 ratios for each of the modifiers in order to see if the change in K_A can be explained by a change in the value of k_{13}.

Kinetics of the reaction as a result of varying modifiers at low fixed substrate concentrations. When substrate concentrations are fixed at the relatively low concentrations of 0.2-0.3 mM, increasing concentrations of modifiers have the effect of increasing the initial velocity of the reaction about three-fold (Figure III.2). This effect is observed for all of the nucleoside triphosphate activators, but as can be seen in the figure, the $MgGTP^{2-}$ and $MgITP^{2-}$ are

TABLE III.1. : Values for K_A, the Michaelis constant for the combination of substrate with the various enzyme-modifier complexes.

Modifier	Weighted Mean[a] K_A (mM)
$MgGTP^{2-}$	0.94 ± 0.06
$MgITP^{2-}$	0.37 ± 0.01
$MgCTP^{2-}$	1.12 ± 0.03
$MgUTP^{2-}$	0.86 ± 0.11
$MgATP^{2-}$ [b]	0.69 ± 0.03

[a]Values for K_A were determined from fits of the data to equation (1).

[b]Value for $MgATP^{2-}$ was determined in Chapter II and is included here for comparative purposes.

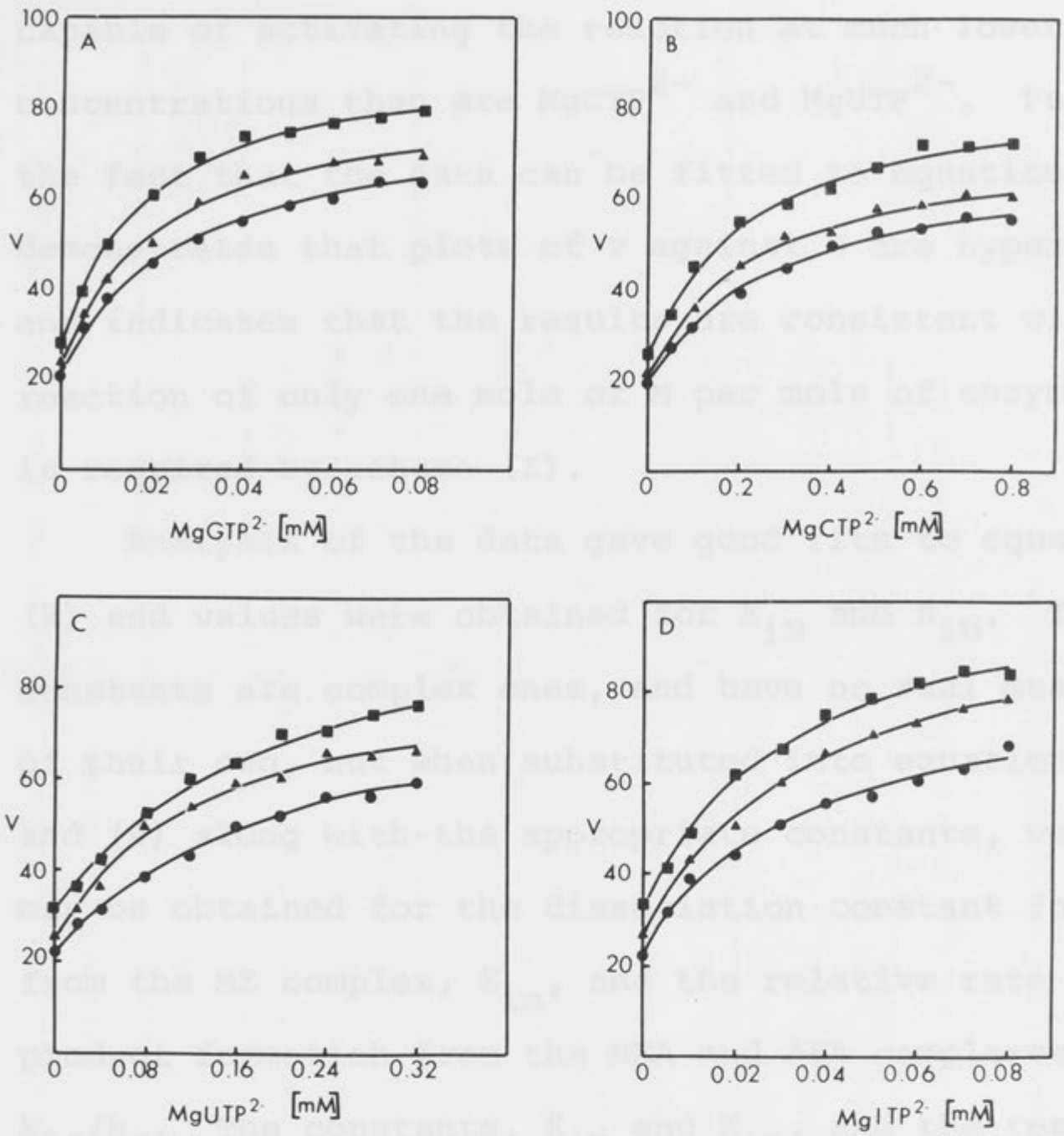


FIGURE III.2. : Effect of varying activator concentrations at fixed concentrations of substrate. In (A), (B), (C) and (D), substrate ($MgIDP^-$) concentrations were 0.20(●), 0.25(▲) and 0.30(■) mM. The lines were drawn from the kinetic constants obtained from fitting the data to equation (2). Initial velocity is expressed as mμmoles of IMP/min/μg of protein.

capable of activating the reaction at much lower concentrations than are $MgCTP^{2-}$ and $MgUTP^{2-}$. Further, the fact that the data can be fitted to equation (2) demonstrates that plots of v against M are hyperbolic and indicates that the results are consistent with the reaction of only one mole of M per mole of enzyme, as is required by scheme (I).

Analysis of the data gave good fits to equation (2) and values were obtained for K_{iN} and K_{iD}. These constants are complex ones, and have no real meaning of their own, but when substituted into equations (3) and (4) along with the appropriate constants, values may be obtained for the dissociation constant for M from the ME complex, K_{im}, and the relative rate of product formation from the MEA and AEA complexes, k_{13}/k_7. The constants, K_{iN} and K_{iD}, and the results of such calculations at three different concentrations of substrate are given in Table III.2, while the overall weighted mean values for K_{im} and k_{13}/k_7 are given in Table III.3.

The results show that K_{im}, the dissociation constant for the ME complex, differs greatly for the different modifiers; $MgGTP^{2-}$ and $MgITP^{2-}$ having a high affinity for the enzyme, while the combinations

TABLE III.2. : True and apparent kinetic constants[a] as determined from results of the activation of the reaction by different activators.

Modifier	Substrate Concentration (mM)	K_{iN} (mM)	K_{iD} (mM)	K_{im} (mM)[b]	k_{13}/k_7[b]	Number of Experiments
$MgGTP^{2-}$	0.20	0.0080 ± 0.0009	0.029 ± 0.003	0.026 ± 0.011	0.64 ± 0.32	2
	0.25	0.0071 ± 0.0009	0.025 ± 0.003	0.021 ± 0.009	0.78 ± 0.36	2
	0.30	0.0062 ± 0.0005	0.020 ± 0.002	0.017 ± 0.007	0.97 ± 0.46	2
$MgCTP^{2-}$	0.20	0.0990 ± 0.0060	0.390 ± 0.027	0.33 ± 0.14	0.62 ± 0.30	8
	0.25	0.0725 ± 0.0068	0.265 ± 0.025	0.22 ± 0.09	0.91 ± 0.44	4
	0.30	0.0681 ± 0.0063	0.206 ± 0.019	0.16 ± 0.07	1.15 ± 0.55	4
$MgUTP^{2-}$	0.20	0.0584 ± 0.0055	0.229 ± 0.028	0.20 ± 0.08	0.64 ± 0.32	2
	0.25	0.0483 ± 0.0058	0.151 ± 0.024	0.13 ± 0.06	0.84 ± 0.42	3
	0.30	0.0628 ± 0.0102	0.215 ± 0.041	0.18 ± 0.08	0.70 ± 0.35	1
$MgITP^{2-}$	0.20	0.0128 ± 0.0013	0.048 ± 0.006	0.054 ± 0.025	0.32 ± 0.15	2
	0.25	0.0101 ± 0.0006	0.040 ± 0.002	0.045 ± 0.018	0.43 ± 0.20	2
	0.30	0.0087 ± 0.0009	0.029 ± 0.003	0.033 ± 0.013	0.54 ± 0.025	2

[a]Values for the apparent constants, K_{iN} and K_{iD}, were determined from fits of the data to equation (1).

[b]Values for the dissociation constant of the enzyme modifier complex (K_{im}) and for the relative rates of product formation from MEA and AEA (k_{13}/k_7) were determined by use of K_{iN} and K_{iD} in equations (3) and (4) along with the appropriate value of K_A (Table III.1), and the values of K_{a1}, K_{a2} and k_3/k_7 reported in Chapter II, Table II.2.

TABLE III.3. : Kinetic constants for the dissociation of the enzyme-modifier complex (K_{im}), the relative rates of product release from the MEA and AEA complexes (k_{13}/k_7), and the ratio of the maximum velocities of the reaction in the presence and absence of a constant ratio of modifier to substrate (V'/V).

Modifier	K_{im} (mM)[a]	k_{13}/k_7[b]	V'/V[c]	k_{13}/k_7[d]
$MgGTP^{2-}$	0.020 ± 0.0005	0.76 ± 0.21	0.86 ± 0.12	0.85 ± 0.42
$MgITP^{2-}$	0.040 ± 0.010	0.39 ± 0.11	0.43 ± 0.06	0.43 ± 0.21
$MgCTP^{2-}$	0.20 ± 0.05	0.79 ± 0.23	0.96 ± 0.15	0.95 ± 0.48
$MgUTP^{2-}$	0.16 ± 0.04	0.71 ± 0.21	0.59 ± 0.06	0.57 ± 0.28
$MgATP^{2-}$[e]	0.040 ± 0.010	0.51 ± 0.16	0.47 ± 0.02	0.46 ± 0.22

[a,b]Values for K_{im} and k_{13}/k_7 are weighted means of those given in Table III.2.

[c]Values for V' and V were obtained by fitting two sets of data, one of which is shown in Figure III.3, to equation (1).

[d]Values for k_{13}/k_7 determined by use of the V'/V values along with the appropriate kinetic constants in equation (5).

[e]Values of the constants for $MgATP^{2-}$ were determined in Chapter II and are included here for purposes of comparison.

for $MgCTP^{2-}$ and $MgUTP^{2-}$ ~~is~~ are 5 and 10 times weaker.

The determination of k_{13}/k_7 gives an indication that the rate of product formation from the E-$MgITP^{2-}$ complex is less than that from the other complexes, but the standard errors (which are accumulated in calculation (3)) of the values are such that no definitive conclusions can be reached. Thus it becomes necessary to determine k_{13}/k_7 by a more direct method in order to determine this ratio.

Kinetics of the reaction when modifier and substrate are varied in constant ratio. In order to facilitate the determination of the maximum velocity of plots in which concentrations of modifier and substrate are varied in constant ratio and also in the absence of modifier, high concentrations of substrate were used. But under these conditions, free Mg^{2+} was present at a concentration of 2.5 times the substrate concentration and it was necessary to make corrections for Mg^{2+} inhibition. In order that this might be done, it was assumed that Mg^{2+} had the same inhibitory effect as when the modifier, $MgATP^{2-}$, was used in constant ratio with substrate, and corrections were made accordingly (cf. Chapter II).

Plots of such corrected data are illustrated in Figure III.3 and show that while all modifiers activated the reaction at lower substrate concentrations, $MgITP^{2-}$ and $MgUTP^{2-}$ had the effect of decreasing the velocity of the reaction at high substrate concentrations, while $MgCTP^{2-}$ and $MgGTP^{2-}$ had less marked effects of reducing the velocity of the reaction at high concentrations.

Comparison of the maximum velocities of the reaction, both in the presence and absence of constant ratios of activators gives a direct measure of k_{13}/k_7 provided that the M/A ratio is large relative to the concentrations of each needed to saturate the enzyme. When this condition is not met, equation (5) must be used to determine the k_{13}/k_7 ratio. Further, if the experimental V'/V value is close to the calculated k_{13}/k_7 ratio, the conditions would be such that the maximum velocity ratio of the reaction would approximate k_{13}/k_7.

Values for the maximum velocities, both in the presence and absence of modifiers, were determined by analyzing the data, and are given in Table III.3. These values were also used in conjunction with equation (5), and it may be seen from Table III.3 that

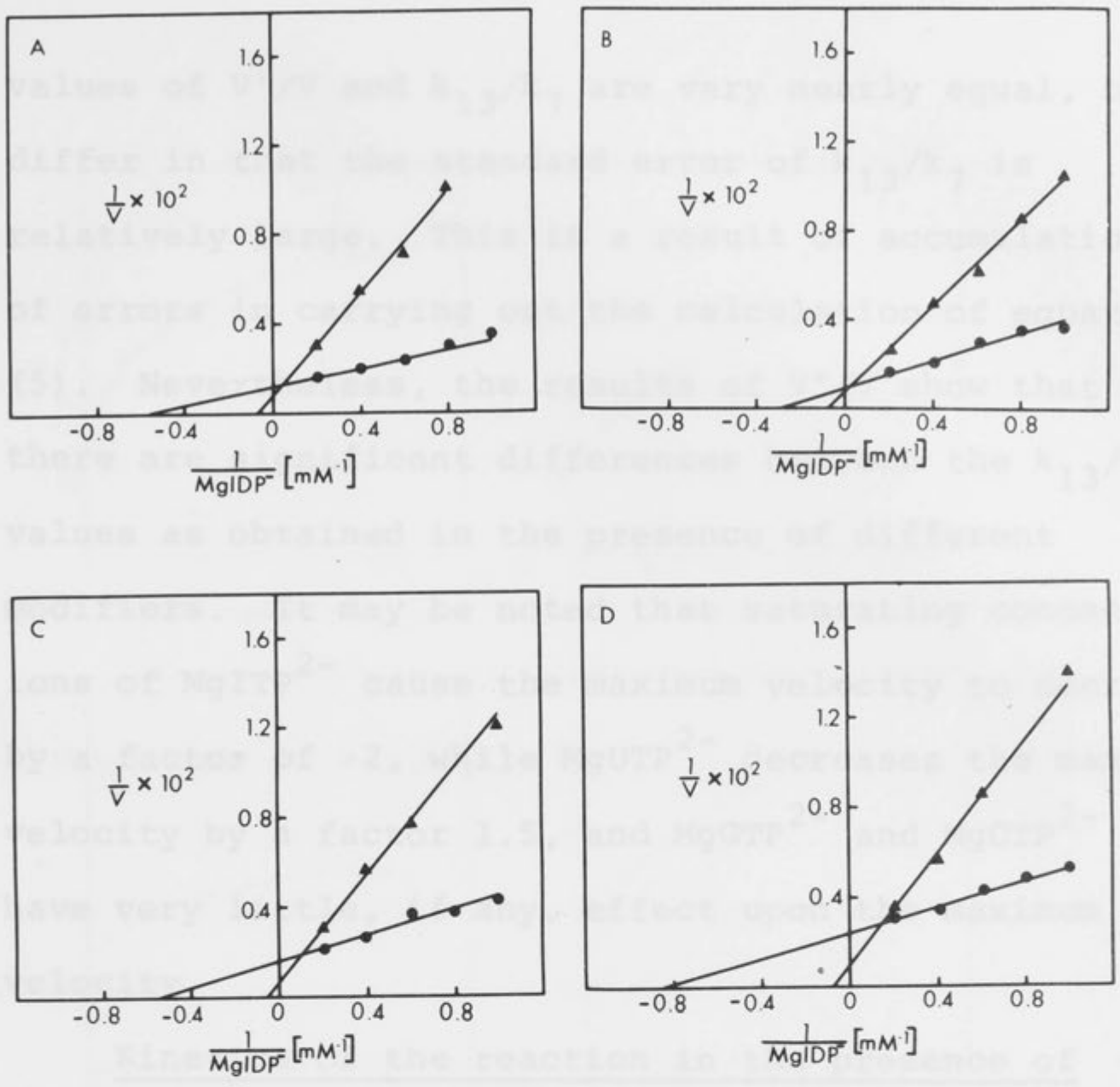


FIGURE III.3. : Effect of maintaining a constant ratio of activator:substrate on the maximum velocity of the reaction. The activators $MgGTP^{2-}$ (A), $MgCTP^{2-}$ (B), $MgUTP^{2-}$ (C) and $MgITP^{2-}$ (D) were present in a constant ratio of 1:1 with substrate concentration. Initial velocity values, both in the presence (●) and absence (▲) of activators, were corrected for inhibition by free Mg^{2+} as described in the text before the data were fitted to equation (1). The lines were drawn from the constants obtained from that fit. Initial velocity is expressed as mμmoles of Pi/min/μg of protein.

values of V'/V and k_{13}/k_7 are very nearly equal, but differ in that the standard error of k_{13}/k_7 is relatively large. This is a result of accumulation of errors in carrying out the calculation of equation (5). Nevertheless, the results of V'/V show that there are significant differences between the k_{13}/k_7 values as obtained in the presence of different modifiers. It may be noted that saturating concentrations of $MgITP^{2-}$ cause the maximum velocity to decrease by a factor of >2, while $MgUTP^{2-}$ decreases the maximum velocity by a factor 1.5, and $MgGTP^{2-}$ and $MgCTP^{2-}$ have very little, if any, effect upon the maximum velocity.

Kinetics of the reaction in the presence of magnesium deoxynucleotide modifiers. Increasing concentrations of magnesium deoxynucleoside triphosphates (MgdNTP) in the presence of a low (0.30) fixed substrate concentration had the effect of increasing the initial velocity in a manner similar to that when MgNTP's are used as modifiers (compare Figures III.2 and III.4). However, a qualitative inspection of the data indicates that higher concentrations of the deoxynucleotides are needed to give equivalent activation to that achieved by nucleotide modifiers.

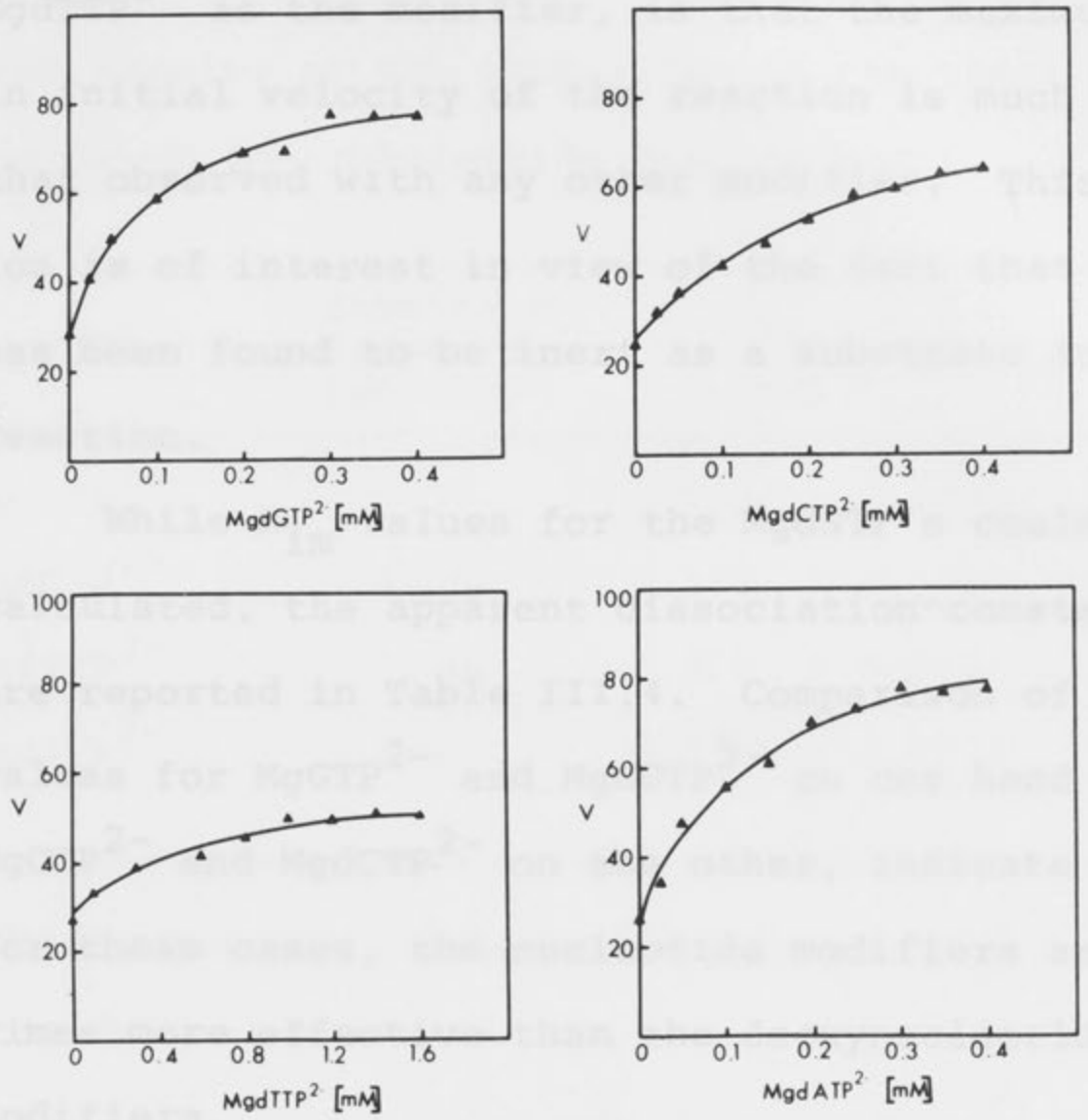


FIGURE III.4. : Effects of deoxynucleotide activators on the initial velocity at fixed concentrations of substrate. Substrate was present at 0.30 mM. Lines were drawn as described in Figure III.2. Initial velocities are expressed as mμmoles of IMP/min/μg of protein.

A further effect which may be noticed when using $MgdTTP^{2-}$ as the modifier, is that the maximum increase in initial velocity of the reaction is much less than that observed with any other modifier. This observation is of interest in view of the fact that $MgdTDP^{-}$ has been found to be inert as a substrate for the reaction.

While K_{im} values for the MgdNTP's could not be calculated, the apparent dissociation constants, K_{iD}, are reported in Table III.4. Comparison of these values for $MgGTP^{2-}$ and $MgdGTP^{2-}$ on one hand and for $MgCTP^{2-}$ and $MgdCTP^{2-}$ on the other, indicate that for these cases, the nucleotide modifiers are 5 and 3 times more effective than the deoxynucleotide modifiers.

This investigation using deoxynucleotide modifiers, could not be further pursued because the cost of deoxynucleotides needed for a complete investigation would be prohibitive (>$2,000).

Discussion

The quantitative conclusions of this investigation rest upon the validity of the proposed reaction scheme. Thus, while all results so far obtained are consistent with the proposed mechanism (Scheme I),

TABLE III.4. : Apparent kinetic constants[a] as determined from results of the activation of the reaction by magnesium deoxynucleoside triphosphates.

Modifier	Substrate Concentration (mM)	K_{iN} (mM)	K_{iD} (mM)
$MgdGTP^{2-}$	0.30	0.0330 ± 0.0058	0.104 ± 0.017
$MgdCTP^{2-}$	0.30	0.173 ± 0.023	0.633 ± 0.104
$MgdTTP^{2-}$	0.30	0.320 ± 0.186	0.660 ± 0.417
$MgdATP^{2-}$	0.30	0.0367 ± 0.0076	0.140 ± 0.027

[a]Values for the constants were determined from fits of the data to equation (2).

it has not been proved to be the actual mechanism. But, by assuming for the time being that the proposed reaction scheme is correct, constants may be calculated to describe the proposed scheme. It must also be emphasized that it is essential to have a proposed reaction scheme before any constants can be calculated, and also in order to interpret results of the type reported in this chapter.

The results of the investigations in this chapter have shown that the modifier site is non-specific, in that both purine and pyrimidine nucleotides as well as nucleotide and deoxynucleotide modifiers, are capable of reacting with the enzyme to cause a similar effect on the reaction. However, higher concentrations of some modifiers are required before equivalent activation is effected.

A comparison of the dissociation constants for the enzyme-modifier complexes (K_{im}) shows that the purine nucleotides, $MgITP^{2-}$, $MgGTP^{2-}$ and $MgATP^{2-}$ (cf. Chapter II) combine the most strongly, while the pyrimidine nucleotides, $MgCTP^{2-}$ and $MgUTP^{2-}$ combine with the enzyme in a much weaker fashion. Further, when $MgdGTP^{2-}$ and $MgdCTP^{2-}$ were used as modifiers, their apparent dissociation constants indicated that

they bound less well than their nucleotide counterparts. Thus it appears that both the base and sugar portions of the modifier play a role in binding to the enzyme.

The value of the Michaelis constant for the combination of substrate (K_A) with the MgITP-E complex is less than those obtained in the presence of the other modifier by a factor of >2. In correlation with this result, it has been found that the relative rate of product formation from the MgITP-E-A and AEA complexes (k_{13}/k_7) is also significantly less than from those containing other modifiers. This result could be interpreted to mean that in the equation, $K_A = \frac{k_{12} + k_{13}}{k_{11}}$, k_{13} is smaller when $MgITP^{2-}$ is the activator than for other enzyme-modifier complexes, and thus, provided that $k_{13} \geq k_{12}$, K_A could also be reduced. In this case, then, an effect which is apparently a binding effect (decrease of Michaelis constant) could, in reality, be the result of a kinetic effect. Such a result must remain speculative, as the relative rates of k_{12} and k_{13} are not known.

A common feature of the MgNTP modifiers is that when present at saturating concentrations, all may reduce the maximum velocity of the reaction, even

though the effect is slight with some of the activators. Quantitative determination of k_{13}/k_7 ratios demonstrates that k_{13} is not a constant for all MEA complexes, as some give rise to products faster than others. The relative rates at which the $MgCTP^{2-}$:$MgGTP^{2-}$:$MgUTP^{2-}$:$MgATP^{2-}$ (Chapter II):$MgITP^{2-}$-enzyme-subtrate complexes break down to give products, are 1.00:0.87:0.61:0.50:0.43.

Summary

The effects of different nucleoside and deoxynucleoside triphosphates on the nucleoside diphosphatase reaction have been studied. All nucleoside and deoxynucleoside triphosphates tested can act as activators of the reaction, although they do so with varying degrees of efficiency.

Dissociation constants for the various enzyme-modifier complexes have been determined, and the enzyme shows the highest affinity for $MgGTP^{2-}$ > $MgITP^{2-}$ > $MgUTP^{2-}$ > $MgCTP^{2-}$.

The maximum velocity of the reaction is decreased by the presence of high concentrations of activators. This effect is most marked with $MgITP^{2-}$ > $MgUTP^{2-}$ > $MgGTP^{2-}$ > $MgCTP^{2-}$.

CHAPTER IV

KINETIC STUDIES OF THE REACTION CATALYZED BY FORMS OF NUCLEOSIDE DIPHOSPHATASE WHICH EXHIBIT LINEAR KINETICS

CHAPTER IV

KINETIC STUDIES OF THE REACTION CATALYZED BY FORMS OF NUCLEOSIDE DIPHOSPHATASE WHICH EXHIBIT LINEAR KINETICS

Introduction

The results reported in previous chapters have indicated that double reciprocal plots of the initial velocity of the nucleoside diphosphatase reaction as a function of substrate concentration are linear with freshly prepared enzyme in the presence of relatively high concentrations of $MgATP^{2-}$ and with an aged enzyme preparation either in the presence or absence of $MgATP^{2-}$. These results contrast with the non-linear kinetics that are observed with freshly prepared (native) enzyme in the absence of the nucleoside triphosphate, and it was pointed out that the possible reasons for obtaining linear kinetics are :

1. Only one molecule of substrate reacts per molecule of enzyme.
2. Two molecules of substrate react, but in an independent manner.
3. Changes in the magnitude of the rate constants are such as to reduce the steady-state concentration of one of the two enzyme-substrate

complexes to levels which are kinetically insignificant under the experimental conditions. The latter point can be illustrated by re-arranging the initial velocity equation (1) of Chapter II in the form

$$v = \frac{V\left(A + \frac{k_3}{k_7}K_{a2}\right)}{A + K_{a2} + \frac{K_{a1}K_{a2}}{A}}$$

from which it is apparent that if k_3 and K_{a1} tend to zero, the equation reduces to that for a single substrate reaction.

Although no definitive conclusions could be reached at this stage as to the reasons for obtaining linear double reciprocal plots with the aforementioned enzymes, nevertheless the opportunity was presented of carrying out product inhibition experiments without the difficulties arising from non-linear double reciprocal plots. It was anticipated that such investigations, as well as results of isotope exchange experiments, may well shed light on the reaction mechanism and contribute to an understanding of the action of the allosteric modifier. In addition, it was of interest to compare the reaction mechanism for an allosteric phosphatase with that deduced for other phosphatases which are not

of the allosteric type.

This chapter reports the results of preliminary investigations on the mechanisms of the reactions catalyzed by the forms of nucleoside diphosphatase which exhibit linear plots of 1/v against $1/MgIDP^-$. As a result of these studies, some information pertaining to the reaction mechanisms has been gained, although more experimental work must be done before definite conclusions can be made as to how the double reciprocal kinetic plots become linear, and in order to fully explain the observed product inhibition patterns in relation to the results of isotope exchange experiments.

Experimental

Materials

The sodium salt of IMP was purchased from P-L Biochemicals, and exhibited a single spot when chromatogramed on DEAE-cellulose paper according to the method of Morrison (1968). DEAE-Cellulose paper (DE-81) was a product of Whatman. IMP-8-C^{14} (29.3 mC/mM) was supplied by Schwarz BioResearch Inc. Carrier-free inorganic phosphate (Pi^{32}) was obtained

from the Australian Atomic Energy Commission.

Methods

In calculating the concentrations of free IMP and Pi to be used as product inhibitors, a stability constant of 100 M^{-1} was assumed for the formation of the MgIMP complex (Walaas, 1958) and one of 76 M^{-1} for the MgPi complex (Smith and Alberty, 1956). Because of the difficulty in distinguishing the inhibitory species, and because of the low concentrations of MgIMP and MgPi compared with the concentrations of the uncomplexed species, free IMP and Pi have been considered as the inhibitors and MgIMP and MgPi have been considered as being inert.

Experimental data from the product inhibition experiments were plotted as a double reciprocal function of initial velocity against substrate concentration, in order to determine the nature of the plot. According to whether the inhibition was competitive, non-competitive or uncompetitive, the data were fitted to equations (1), (2) or (3) with the computer programs of Cleland (1963d), in order to determine values for the kinetic constants.

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A} \qquad (1)$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \qquad (2)$$

$$v = \frac{VA}{K + A\left(1 + \frac{I}{K_{ii}}\right)} \qquad (3)$$

These equations describe inhibition patterns in which the slope and/or intercept differences are a linear function of inhibitor concentration. If there was doubt as to whether particular sets of data were best described by equations (1) or (2) on one hand, or by equations (2) or (3) on the other, they were fitted to both of the equations in question. The analysis which gave the smaller variance was taken to be the actual inhibition pattern.

Isotope exchange experiments were carried out at both pH 5.5 and 8.5 in 0.1 M acetate and 0.1 M triethanolamine buffer, respectively. Substrate ($MgIDP^-$) was present at a concentration of 1.2 mM, free Mg^{2+} was maintained at 2.5 mM, and when present, $MgATP^{2-}$ was used at a concentration of 2.0 mM. The products,

IMP and/or Pi, were added at concentrations of 5.0 mM along with the appropriate radioactive compounds to make a total volume of 0.30 ml. Each tube contained 2-3 x 10^6 cpm of Pi^{32} when Pi was added as the product inhibitor and 2-3 x 10^5 cpm of IMP-8-C^{14} when IMP was added as product inhibitor. A zero time sample (0.03 ml) was taken just before the reaction was started by adding 0.23 µg of protein to the remaining 0.27 ml of reaction mixture; 0.03 ml samples of the reaction mixture were taken at appropriate times with a Hamilton microlitre syringe and spotted on DEAE-cellulose paper. This had the effect of immediately stopping the enzymatic action. Nucleotides and Pi were separated on the DEAE-cellulose paper according to the method of Morrison (1968), which consisted of developing the papers for 4 hr in 0.6 M ammonium formate, pH 3.1. Following chromatography in this system, the radioactive spot of Pi^{32} was located by autoradiography on X-ray film and was found to run just ahead of, and slightly overlapping, the IMP spot. IDP was well separated from, and ran very much slower than IMP. After chromatography, the papers were dried and the IDP spots were located under UV light, and excised

from the paper, with care being taken to ensure that each spot removed had equivalent areas of the DEAE-cellulose paper. The papers containing the IDP spots were fluted and placed in vials containing 15 ml of toluene based scintillation fluid, and counted in a Packard model 3003 scintillation counter (cf. Morrison, 1968).

Results

Elucidation of the reaction mechanism (order of product release) of nucleoside diphosphatase, requires that product inhibition studies be carried out on both the native and aged forms of enzyme. These studies are impractical with the native enzyme, as very extensive data would be required when non-linear kinetic plots[1] are obtained. Further, as was discussed in the Introduction to this thesis, velocity equations and methods for quantitative analysis of such data have not yet been developed. These problems can be by-passed in the first instance by making a study of the kinetics of the native enzyme in the presence of

---

[1] In this chapter, the term "kinetic plots" will be used to refer to double reciprocal plots of initial velocity against substrate ($MgIDP^-$) concentration.

high concentrations of the activator, $MgATP^{2-}$, and of the aged enzyme both in the absence and presence of activator. In these instances, the kinetic plots are all linear.

The results of Chapter II suggested that with the native enzyme, $MgATP^{2-}$ may combine at one of two inter-dependent substrate sites, so that at sufficiently high concentrations of this nucleotide, relative to its dissociation constant, only one substrate site is free to interact with substrate. This situation, whereby only one catalytic site is reactive, would then give rise to linear kinetic plots.

In studying the product inhibition patterns for nucleoside diphosphatase, three products of the reaction namely, Mg^{2+}, IMP and Pi, have been considered. Magnesium has been considered as a product, as hydrolysis of $MgIDP^{-}$ to IMP results in the dissociation of Mg^{2+} under the conditions of the enzymatic assay. This makes the reaction Uni Ter in the nomenclature of Cleland (1963a).

Studies on the mechanism of the reaction catalyzed by the native enzyme in the presence of $MgATP^{2-}$

To maintain conditions whereby the native enzyme

would exhibit linear kinetics and could be considered to be saturated with $MgATP^{2-}$, in all experiments carried out with this enzyme, the activator was maintained at a 1:1 (or 1:3) ratio with substrate. Under these conditions, and because $MgATP^{2-}$ combines much better than substrate, the enzyme may be considered as being saturated with $MgATP^{2-}$ even when the substrate concentration is extrapolated to infinity (1/A = 0). Thus, in this study, the native enzyme may be considered as being all in the ME form, with substrate able to react at only one site, forming an MEA complex.

Product inhibition and isotope exchange studies.

The product inhibition patterns which are illustrated in Figure IV.1, demonstrate that non-competitive inhibition patterns are obtained with each of the product inhibitors, Pi, IMP and Mg^{2+}. Furthermore, the slope and intercept changes are a linear function of the increase in inhibitor concentration (linear non-competitive inhibition). These patterns are not consistent with any simple scheme for either an ordered or random release of products, and do not permit any conclusions to be reached as to the order of product release.

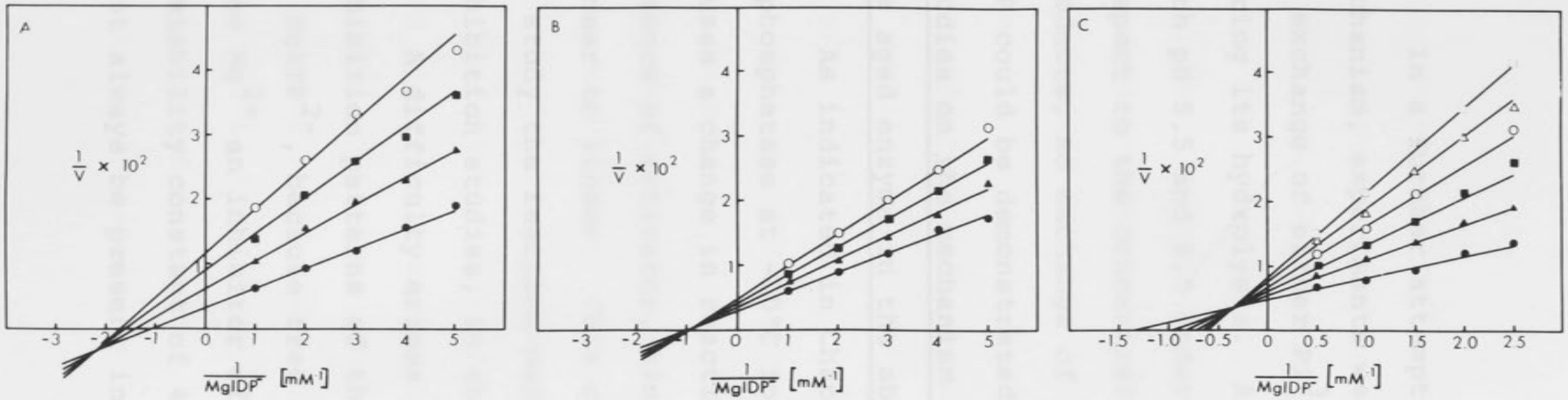


FIGURE IV.1. : Product inhibition of the initial velocity of the reaction catalyzed by the native enzyme in the presence of $MgATP^{2-}$. The concentrations of inhibitors were : (A) Pi; 0(●), 3(▲), 6(■) and 9(O) mM. (B) IMP; 0(●), 3(▲), 6(■) and 9(O) mM. (C) Mg^{2+}; 5(●), 15(▲), 25(■), 35(O), 45(Δ) and 55(□) mM. The ratio of $MgATP^{2-}$:$MgIDP^-$ in (A) and (B) was 1:1, while in (C) the ratio was 3:1. Pi formation was measured in (B) and (C) while IMP formation was measured in (A). Initial velocities are expressed as mμmoles/min/μg of protein. Lines are drawn by using the kinetic constants obtained by fitting the data to equation (2).

In a further attempt to elucidate the reaction mechanism, experiments were carried out to determine if exchange of either Pi^{32} or IMP-8-C^{14} into IDP occurred during its hydrolysis. Although studies were made at both pH 5.5 and 8.5 under a variety of conditions with respect to the concentrations of both substrate and products, no exchange of either Pi^{32} or IMP-8-C^{14} into IDP could be demonstrated.

Studies on the mechanism of the reaction catalyzed by the aged enzyme in the absence of $MgATP^{2-}$

As indicated in Chapter I, storage of nucleoside diphosphatase at -10°C for periods of four months, causes a change in reaction kinetics so that in the absence of activator, kinetic plots change from non-linear to linear. This change offers an opportunity to study the reaction mechanism, as deduced by product inhibition studies, in the absence of activators.

A difficulty arises in studying the product inhibition patterns of the aged enzyme in the absence of $MgATP^{2-}$, because free IDP^{3-} is an activator, and free Mg^{2+} an inhibitor of the reaction. As $MgIDP^{-}$ has a stability constant of 4,000 M^{-1}, Mg^{2+} and/or IDP^{3-} must always be present in the reaction mixture. In

order to prevent Mg^{2+} from becoming inhibitory and thus interfering with the product inhibition patterns obtained with Pi and IMP, in those instances IDP^{3-} was maintained at the relatively high concentration of 0.2 mM which is sufficient to cause activation of the reaction. Conversely, when Mg^{2+} is used as the product inhibitor, the level of free IDP^{3-} is low so that no activation takes place. A further difficulty that could arise in connection with the above product inhibitions would be the combination of products at the empty modifier site to give inhibition.

Because of the above difficulties, these results must be considered as preliminary until further experiments can be done to determine if the effects discussed above do influence the product inhibition patterns.

Product inhibition and isotope exchange studies. The results of inhibition studies carried out on the aged enzyme in the absence of $MgATP^{2-}$ using Pi, IMP and Mg^{2+} as product inhibitors (Figure IV.2), are similar to those obtained for the native enzyme plus $MgATP^{2-}$, in that three linear non-competitive inhibition patterns are obtained. Again, as a result of these patterns, no conclusions may be reached with respect

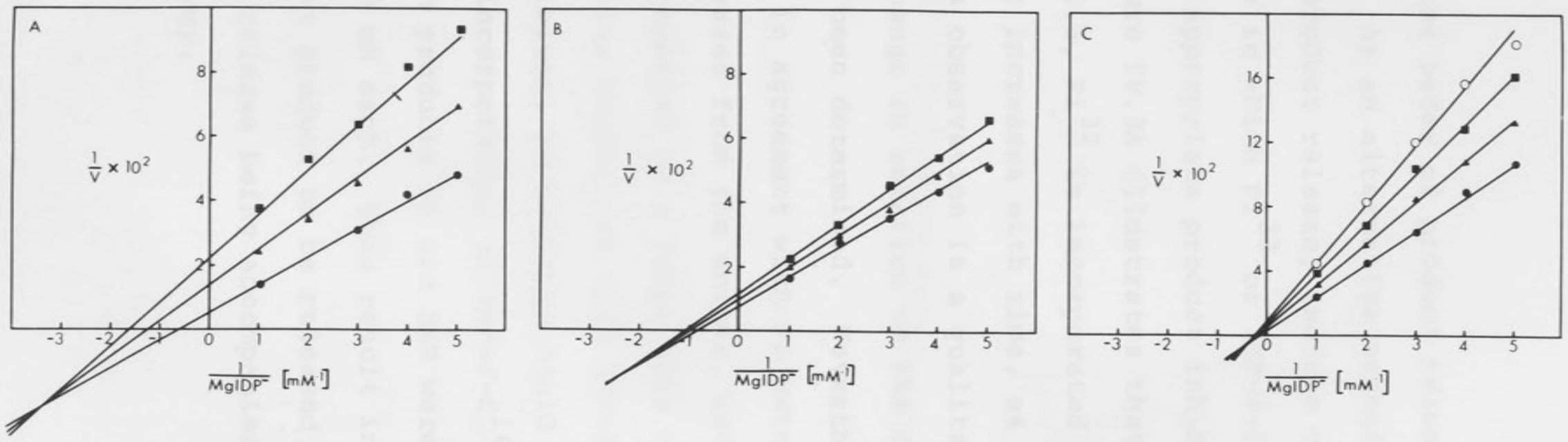


FIGURE IV.2. : Product inhibition of the initial velocity of the reaction catalyzed by the aged enzyme in the absence of $MgATP^{2-}$. Concentrations of inhibitors were : (A) Pi; 0(●), 15(▲) and 30(■) mM. (B) IMP; 0(●), 5(▲) and 10(■) mM. (C) Mg^{2+}; 5(●), 10(▲), 15(■) and 20(O) mM. IMP formation was measured in (A) and (C), while Pi formation was measured in (B). Initial velocities are expressed as mμmoles/min/μg of protein. Lines are drawn by using the kinetic constants obtained by fitting the data to equation (2).

to the order of product release.

As an alternative method for determining the order of product release, isotope exchange studies were done in which Pi^{32} or IMP-8-C^{14} was added along with the appropriate product inhibitor of the reaction. Figure IV.3A illustrates that, in acetate buffer at pH 5.5, Pi^{32} is incorporated into IDP, and that this rate increases with time, as IDP is being hydrolyzed. This observation is a qualitative one and the rate of exchange in relation to the initial velocity has not yet been determined. Nevertheless, the above results are in agreement with Pi being the first product to be released from the enzyme, and that this step is not accompanied by a large loss of free energy. Under similar conditions with IMP-8-C^{14} as product inhibitor, no exchange could be demonstrated. Further, no incorporation of IMP-8-C^{14} was observed even when both products Pi and IMP were present at concentrations of 5 mM each. This result indicates that IMP is not the first product to be released, and is consistent with IMP release being accompanied by a large loss in free energy.

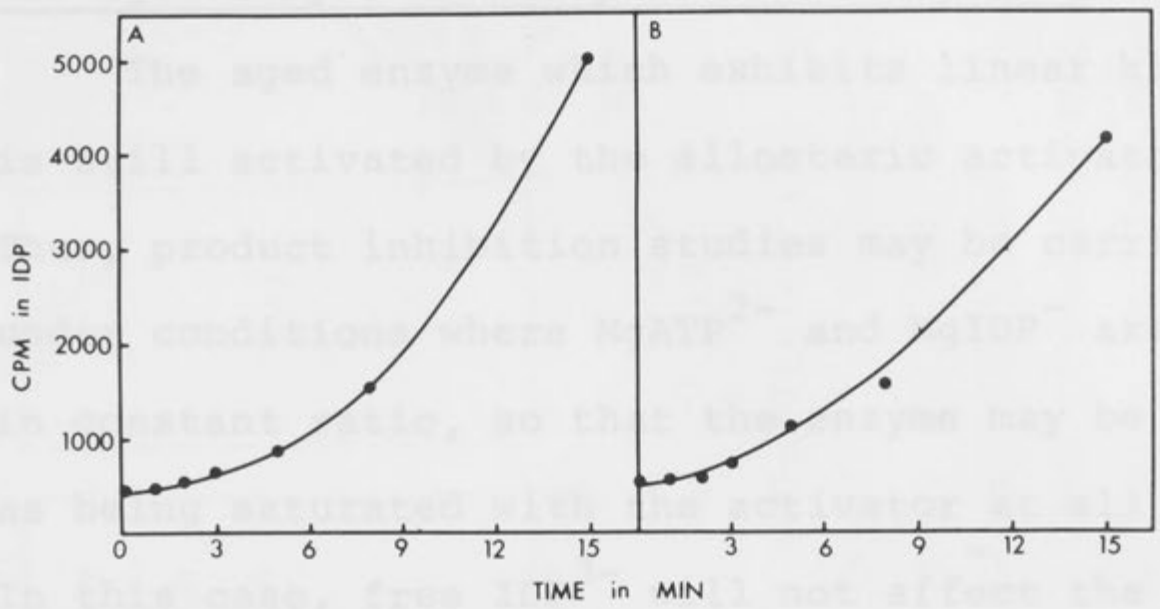


FIGURE IV.3. : Exchange of Pi^{32} into IDP during hydrolysis of IDP. (A) Exchange into IDP as catalyzed by the aged enzyme in the absence of $MgATP^{2-}$. Exchange was carried out at pH 5.5 as described in the text. Initial velocity was maintained for about 4 minutes. (B) Exchange into IDP as catalyzed by the aged enzyme in the presence of 2.0 mM $MgATP^{2-}$. Exchange was carried out at pH 5.5 as described in the text. Initial velocity was maintained for about 3 minutes. Lines are drawn by eye.

Studies on the mechanism of the reaction catalyzed by the aged enzyme in the presence of $MgATP^{2-}$

The aged enzyme which exhibits linear kinetic plots is still activated by the allosteric activator, $MgATP^{2-}$. Thus, product inhibition studies may be carried out under conditions where $MgATP^{2-}$ and $MgIDP^{-}$ are varied in constant ratio, so that the enzyme may be considered as being saturated with the activator at all times. In this case, free IDP^{3-} will not affect the reaction, as it presumably reacts at the same site as $MgATP^{2-}$ and is excluded by the high concentrations of $MgATP^{2-}$.

Product inhibition and isotope exchange studies.

Inhibition patterns obtained with the product inhibitors, Pi, IMP and Mg^{2+}, were non-competitive, non-competitive and competitive, respectively (Figure IV.4). These results are consistent with Mg^{2+} being the last product to leave the enzyme, but give no information pertaining to the order in which Pi and IMP are released. In order to determine which of these products is released from the enzyme first, isotope exchange experiments such as those described previously, were carried out. Figure IV.3B indicates that Pi^{32} is incorporated into IDP in the absence of IMP, which result is consistent with Pi

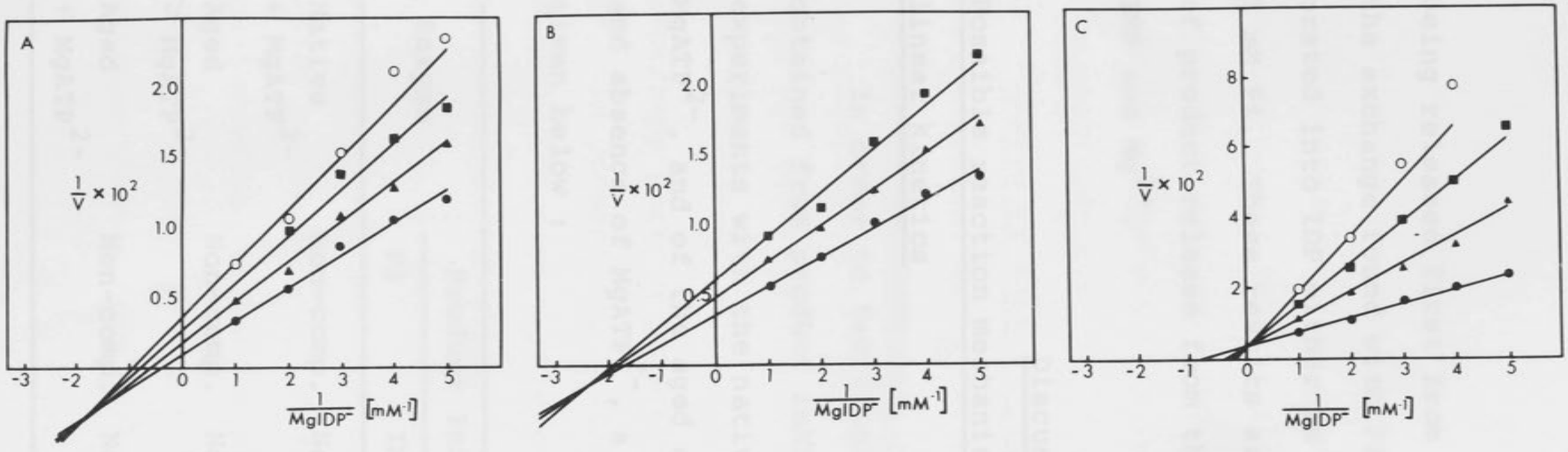


FIGURE IV.4. : Product inhibition of the initial velocity of the reaction catalyzed by the aged enzyme in the presence of $MgATP^{2-}$. Concentrations of inhibitors were : (A) Pi; 0(●), 3(▲), 6(■) and 9(O) mM. (B) IMP; 0(●), 5(▲) and 10(■) mM. (C) Mg^{2+}; 5(●), 20(▲), 35(■) and 50(O) mM. In all experiments the ratio of $MgATP^{2-}$:$MgIDP^-$ was 2:1. IMP formation was measured in (A) and (C) while Pi formation was measured in (B). Initial velocities are expressed as mμmoles/min/μg of protein. Lines in (A) and (B) are drawn by using the constants obtained by fitting the data to equation (2), and the lines in (C) are drawn by using the constants obtained by fitting the data to equation (1).

being released first from the enzyme. In contrast to the exchange found with Pi^{32}, IMP-8-C^{14} was not incorporated into IDP either in the presence or absence of 5 mM Pi. These results are consistent with the order of product release from this form of enzyme being Pi, IMP and Mg^{2+}.

Discussion

Possible reaction mechanisms for the enzymes exhibiting linear kinetics

In order to facilitate discussion of the results obtained from product inhibition and isotope exchange experiments with the native enzyme in the presence of $MgATP^{2-}$, and of the aged enzyme both in the presence and absence of $MgATP^{2-}$, a summary of the results is given below :

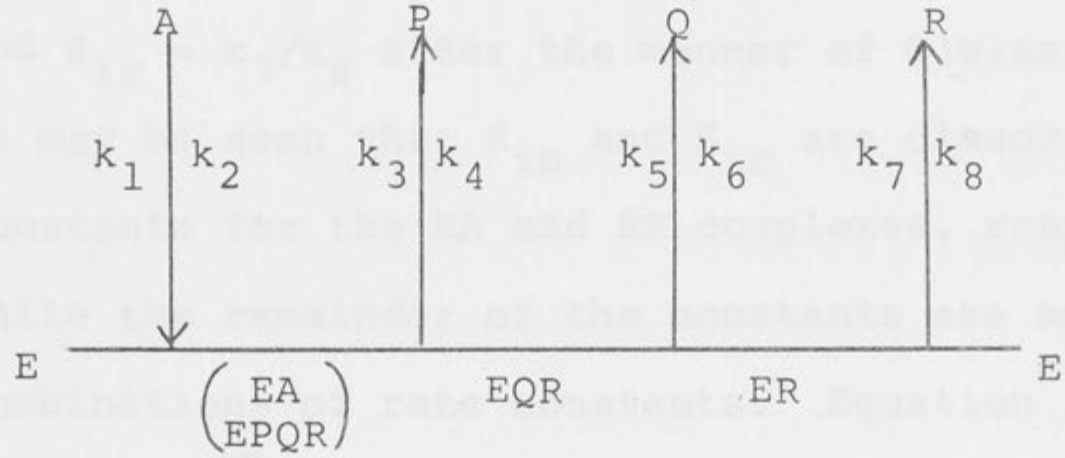
Enzyme	Product Inhibition Patterns			Isotope Exchange
	Pi	IMP	Mg^{2+}	
Native + $MgATP^{2-}$	Non-comp.	Non-comp.	Non-comp.	None
Aged - $MgATP^{2-}$	Non-comp.	Non-comp.	Non-comp.	Present Pi^{32} only
Aged + $MgATP^{2-}$	Non-comp.	Non-comp.	Comp.	Present Pi^{32} only

The results of the above investigations on the reaction mechanisms of the different forms of nucleoside diphosphatase must be considered as being preliminary and as such do not furnish a detailed description of the reaction mechanism. Thus, more experiments must be carried out before this investigation can be considered as being complete.

Random release of products. Despite the limitations of the present data, certain mechanisms may be eliminated and some limited conclusions which pertain to the reaction mechanism may be reached. The use of kinetic data for the elimination of reaction mechanisms which are not in agreement with the experimental data, plays an important part in proposing the actual reaction mechanism. In this connection, a steady-state mechanism which permits the random release of products may be eliminated, as this reaction mechanism would give three non-competitive product inhibition patterns, in which replots of slopes or intercepts against inhibitor concentration would be non-linear. Similarly, a rapid-equilibrium random mechanism can be eliminated because each product inhibitor would give a competitive inhibition pattern. Because these random mechanisms are unlikely to be able

to account for the experimental results with nucleoside diphosphatase, it is necessary to consider an ordered release of products to account for the experimental results.

Ordered release of products. The simplest ordered steady-state scheme for a Uni Ter reaction is shown in scheme (I).



(I)

where E represents free enzyme, and EA and EPQR represent the enzyme-substrate and enzyme-product complexes, respectively. P, Q and R represent the first, second and third products released, and EQR and ER represent enzyme-product complexes. The overall steady-state rate equation for this reaction scheme is :

$$v = \frac{V_1V_2A - {}^{V_1V_2PQR}/_{K_{eq}}}{K_aV_2 + V_2A + \frac{K_aV_2P}{K_{ip}} + \frac{K_aV_2R}{K_{ir}} + \frac{K_pV_1QR}{K_{eq}} + \frac{K_rV_1PQ}{K_{eq}} + \frac{V_1PQR}{K_{eq}} + \frac{K_aV_2AP}{K_{ia}K_{ip}} + \frac{K_rV_1APQ}{K_{ia}K_{eq}} + \frac{V_2AQ}{K_{iq}} + \frac{K_qV_1PR}{K_{eq}}} \quad (4)$$

in which the equilibrium and kinetic constants are defined as : $K_{eq} = \frac{k_1k_3k_5k_7}{k_2k_4k_6k_8}$, $V_1 = \frac{k_3k_5k_7e_t}{k_3k_5 + k_3k_7 + k_5k_7}$,

$V_2 = k_2e_t$, $K_a = \frac{k_5k_7(k_2 + k_3)}{k_1(k_3k_5 + k_3k_7 + k_5k_7)}$, $K_p = \frac{k_2 + k_3}{k_4}$,

$K_q = k_2/k_6$, $K_r = k_2/k_8$, $K_{ia} = k_2/k_1$, $K_{ip} = \frac{k_5(k_2 + k_3)}{k_2k_4}$

and $K_{ir} = k_7/k_8$ after the manner of Cleland (1963a). It may be seen that K_{ia} and K_{ir} are dissociation constants for the EA and ER complexes, respectively, while the remainder of the constants are more complex combinations of rate constants. Equation (4) reduces to : $v = \frac{VA}{K_a + A}$ in the absence of products.

The product inhibition patterns for scheme (I), which is described by equation (4), may be determined by setting equal to zero all terms which contain products other than that added as the product inhibitor.

Thus when P is added as product inhibitor, the reciprocal form of the equation becomes :

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{P}{K_{ip}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{K_aP}{K_{ia}K_{ip}}\right) \qquad (5)$$

As both the slope $\left(\frac{K_a}{V}\right)$ and intercept $\left(\frac{1}{V}\right)$ terms of a double reciprocal plot of 1/v against 1/A are affected

by the presence of P, non-competitive inhibition would be observed, and replots of slopes or intercepts against inhibitor concentration would be linear (linear non-competitive inhibition). When Q is present as product inhibitor, the reciprocal initial velocity equation is :

$$\frac{1}{v} = \frac{K_a}{V}\cdot\frac{1}{A} + \frac{1}{V}\left(1 + \frac{Q}{K_{iq}}\right) \qquad (6)$$

which is an equation describing linear uncompetitive inhibition, as only the intercept $\left(\frac{1}{V}\right)$ term will vary as a function of the concentration of Q. When R, the final product to be released from the enzyme is added, the reciprocal initial velocity equation becomes :

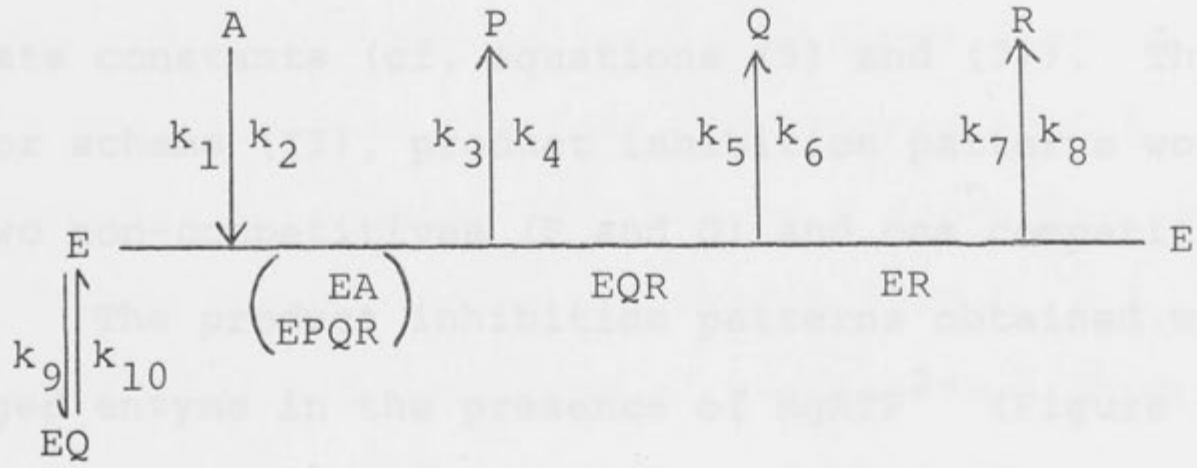
$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{R}{K_{ir}}\right)\frac{1}{A} + \frac{1}{V} \qquad (7)$$

As only the slope $\left(\frac{K_a}{V}\right)$ of a double reciprocal plot will be affected, linear competitive inhibition will result.

The reaction mechanism described by scheme (I) cannot account for any of the three sets of results obtained with the enzymes discussed in Results. Thus consideration was given to possibilities which could affect inhibition patterns in a Uni Ter reaction. One such possibility would be the presence of a dead-end

inhibition, and the effects of such are discussed below.

Effect of an E-IMP dead-end inhibition on product inhibition patterns. If Q were to combine with E so as to form a dead-end complex, the reaction scheme would be written as in scheme (II) :



(II)

Although the velocity equation describing scheme (II) is not given, it has been derived, and differs from that for scheme (I) only in the addition of Q, PQ and PQ^2 terms in the denominator. The PQ and PQ^2 terms are of no consequence when only one product is added as inhibitor, while the additional term in Q has the effect of changing the product inhibition pattern for Q from uncompetitive to non-competitive, as described by equation (8) :

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{Q}{K_{Iq}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{Q}{K_{iq}}\right) \qquad (8)$$

where $K_{Iq} = k_{10}/k_9$, the dissociation constant for the EQ dead-end complex, and K_{iq} retains the same meaning as described below equation (4).

Product inhibition patterns for P and R as products would be the same as in scheme (I), and the kinetic constants would retain the same meanings in terms of rate constants (cf. equations (5) and (7)). Thus for scheme (II), product inhibition patterns would be two non-competitives (P and Q) and one competitive (R).

The product inhibition patterns obtained with the aged enzyme in the presence of $MgATP^{2-}$ (Figure IV.4) are in accord with the product inhibition patterns expected for scheme (II), and are consistent with Mg^{2+} being the final product to be released. However, no conclusions can be reached from the kinetic data as to the order of Pi and IMP release. But an answer to this problem comes from isotope exchange experiments, which by demonstrating the exchange of Pi^{32} into IDP in the absence of IMP, indicates that Pi is the first product to leave the enzyme. Thus the results are consistent with the order of product release from the aged enzyme plus $MgATP^{2-}$ being Pi, IMP and Mg^{2+}.

In connection with the results illustrated in Figure IV.4A, it is of interest to note that the lines

intersect at a point below the 1/v axis. It may be shown that this intersection point is equal to $\frac{1}{V}\left(1 - \frac{K_a}{K_{ia}}\right)$ which may be negative only when $K_a > K_{ia}$. The constants obtained by quantitative analysis of the data, including those of Figure IV.4, are reported in Table IV.1. It may be seen from these figures that K_a, the Michaelis constant, is greater than K_{ia}, the dissociation constant for EA, by a factor of about three, and thus the Michaelis assumption that $K_a = K_{ia}$ is invalid for the aged form of nucleoside diphosphatase in the presence of activator. Such a result is similar to results for a number of other enzymes (e.g. Hsu et al., 1966; Bridger and Cohen, 1968; Soldin and Balinsky, 1968).

Although scheme (II) can account for the results of the aged enzyme in the presence of $MgATP^{2-}$, the results from the native enzyme in the presence of $MgATP^{2-}$ and from the aged enzyme in the absence of activator cannot be accounted for with this scheme, as three non-competitive inhibition patterns are obtained. However, such results can be explained by assuming that in addition to the dead-end inhibition postulated in scheme

TABLE IV.1. : Values of the kinetic constants[a] for the reaction of Pi, IMP and Mg^{2+} with the aged enzyme in the presence of $MgATP^{2-}$.

Product Inhibitor	Kinetic Constant[b]	Weighted Mean Value of Kinetic Constants (mM)	Number of Experiments
Pi	K_{ia}	0.22 ± 0.06	3
	K_{ip}	9.27 ± 1.11	3
IMP	K_{iq}	15.0 ± 1.73	3
	K_{Iq}	16.9 ± 2.06	3
Mg	K_{ir}	10.2 ± 0.48	3
-	K_{a}	0.60 ± 0.02	9

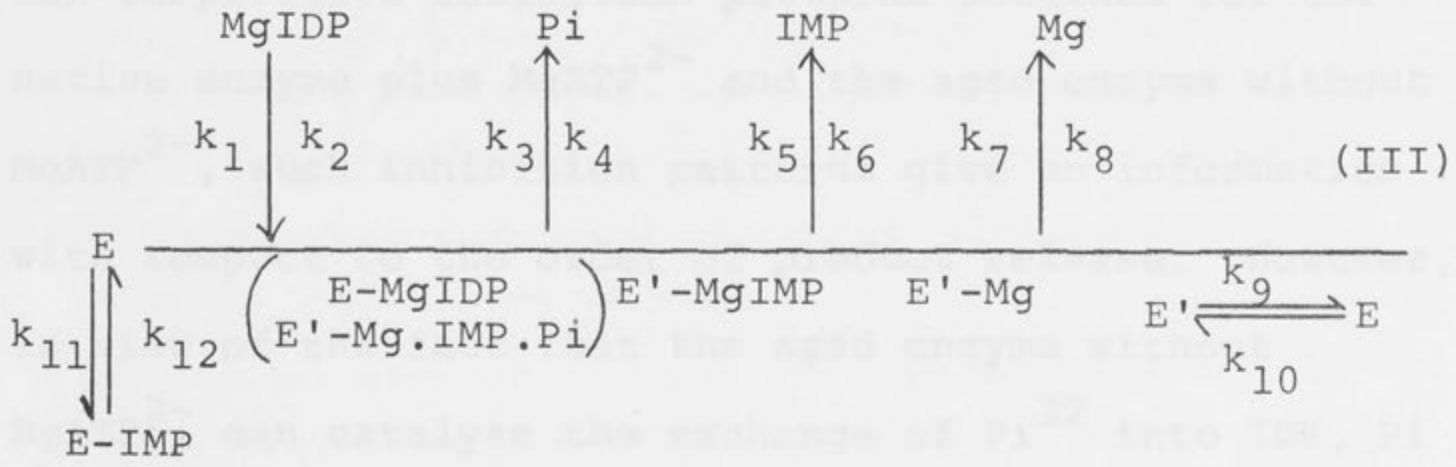
[a]Values for kinetic constants were obtained by fitting the data, including those of Figure IV.1, to equations (1) and (2).

[b]The meaning of the kinetic constants, in terms of rate constants, is as described under equation (4).

(II), an isomerization of the free enzyme, E, takes place, and the effects of such an isomerization are discussed below.

Effect of an isomerization of free enzyme on the product inhibition patterns. If in a reaction mechanism the free enzyme, E, can exist in two forms, E and E', the product inhibition patterns are different from the mechanism in which E exists in only one form. However, it should be noted that isomerization of the EA, EPQR, EQR or ER forms of enzyme would not change the product inhibition patterns, but would change the meanings of the kinetic constants.

Thus in the scheme (III) :



E' represents an isomeric form of enzyme which must revert to E before substrate may combine. All product inhibition patterns for scheme (III) would be linear

non-competitive, and as such could account for the results from both the native enzyme in the presence of $MgATP^{2-}$ (Figure IV.1) and the aged enzyme in the absence of $MgATP^{2-}$ (Figure IV.2).

The overall rate equation for scheme (III) has been derived, but as a result of the isomerization step, all kinetic constants become complex ones and as such, contribute little to an understanding of the reaction mechanism. Thus, it has not been included in this chapter, and the inhibition constants reported for the native enzyme and for the aged enzyme in the absence of activator in Table IV.2 are apparent ones.

Although scheme (III) can account for the three non-competitive inhibition patterns obtained for the native enzyme plus $MgATP^{2-}$ and the aged enzyme without $MgATP^{2-}$, such inhibition patterns give no information with respect to the order of product release. However, in view of the fact that the aged enzyme without $MgATP^{2-}$ can catalyze the exchange of Pi^{32} into IDP, Pi may be considered as the first product. As all three enzymes catalyze the same reaction, presumably in a similar fashion, it is not unreasonable to suppose that the release of products could be Pi, then IMP and

TABLE IV.2. : Comparison of the apparent kinetic constants[a] for the reaction of Pi, IMP and Mg^{2+} with both the aged and native enzymes.

Product Inhibitor	Kinetic Constant	Weighted Mean Values of Kinetic Constants (mM)		
		Aged Enzyme plus Activator	Native Enzyme plus Activator	Aged Enzyme No Activator
Pi	K_{ii}	3.43 ± 0.77 (3)	3.59 ± 0.24 (3)[b]	9.04 ± 1.03 (3)
	K_{is}	9.27 ± 1.11 (3)	7.23 ± 0.52 (3)	48.8 ± 8.71 (3)
IMP	K_{ii}	15.0 ± 1.73 (3)	14.4 ± 1.88 (3)	13.4 ± 1.80 (6)
	K_{is}	16.9 ± 2.06 (3)	11.6 ± 0.88 (3)	55.3 ± 13.3 (6)
Mg^{2+}	K_{ii}	-	58.5 ± 8.89 (1)	6.02 ± 2.58 (3)
	K_{is}	10.2 ± 0.48 (3)	11.9 ± 1.38 (1)	14.9 ± 1.38 (3)
-	K_a	0.60 ± 0.02 (3)	0.81 ± 0.02 (7)	3.86 ± 1.17 (3)[c]

[a]Values for the kinetic constants were obtained by fitting the data, including that of Figures IV.1, IV.2 and IV.4 to equations (1) and (2).

[b]The figure in brackets indicates the number of experiments from which the kinetic constants were calculated.

[c]This value was calculated only from plots where Mg^{2+} was added as the product inhibitor, as activation by IDP^{3-} when Pi and IMP were added as product inhibitors results in only apparent K_a values being obtained.

finally Mg^{2+} for all the enzymes.

Although scheme (III) can explain the three non-competitive product inhibition patterns obtained for the native enzyme plus activator, other possible explanations for such results in the aged enzyme without activator, have not yet been excluded. One such possibility is the reaction of product inhibitors at the vacant activator site in order to cause inhibition. It appears that such a combination could take place with Mg^{2+}, as the effect of Mg^{2+} on the intercept of kinetic plots (K_{ii} in Table IV.2) increases about eight-fold in the absence of activator. It may be possible to test the above hypothesis by determining product inhibition patterns in the presence of a competitive inhibitor with respect to $MgATP^{2-}$, which has been shown not to interfere with substrate hydrolysis. If this were done, product inhibitors could not react at the activator site to cause inhibition.

General consideration of the proposed reaction mechanisms. The product inhibition and isotope exchange studies carried out on nucleoside diphosphatase have shown that the reaction mechanism is not a simple one. Thus, in order to account for the results in the

simplest manner, it has been necessary to postulate the existence of isomeric forms of the free enzyme and/or the formation of a dead-end complex. If it is assumed that the order of product release for all three forms of enzyme is the same, the general reaction mechanism may be illustrated by scheme (III) for the native enzyme plus $MgATP^{2-}$ and for the aged enzyme minus $MgATP^{2-}$ while for the aged enzyme plus $MgATP^{2-}$, the reaction mechanism is the same, except that the $E' \longrightarrow E$ reaction could be considered as being fast relative to other rate constants, so that E' is not present in kinetically significant amounts. Thus in this instance, the modifier may act by increasing the rate of a step in the reaction, thereby causing a change in both the catalytic properties and the observed product inhibition patterns.

The formation of an E-IMP dead-end inhibition has been formulated in the reaction schemes for all three enzymes. However, in practice, it is not possible to distinguish between E-IMP or E'-IMP dead-end formation, as both will give the same inhibition patterns. The formation of an E-IMP complex has been used in preference to the E'-IMP form, as it appears that E' is not

present in significant amounts in the aged enzyme plus $MgATP^{2-}$, while it is still necessary to postulate an E-IMP dead-end complex to account for the results.

While the results of the aged enzyme plus activator are in agreement with the scheme (III), containing only a dead-end inhibitor, isomerization of the free enzyme form is necessary to account for the results of both the native enzyme in the presence of $MgATP^{2-}$ and of the aged enzyme without activator. Isomerizations have been postulated for a number of phosphatases (cf. Hsu et al., 1966), but in those cases, the data are consistent with the isomerization taking place within the enzyme-Pi complex, rather than in the free enzyme, as seems to be the case for nucleoside diphosphatase. Thus in this respect, nucleoside diphosphatase differs from other phosphatases.

A further point of comparison between nucleoside diphosphatase and phosphatases which are not allosteric, is the order in which products are released. Thus, for the reactions catalyzed by glucose-6-phosphatase (Hass and Byrne, 1960), potato phosphomonoesterase (Hsu, Anderson and Cleland, 1966), phosphoserine phosphatase (Byrne, 1961) and adenosine triphosphatase

(Alberts, Fahn and Koval, 1963), it has been shown that the dephosphorylated substrate is released first with the subsequent formation of a high energy enzyme-Pi complex, the breakdown of which is the rate-limiting step of the reaction. A similar enzyme-Pi complex was postulated for nucleoside diphosphatase (Chapter I, p.77), when it was found that the maximum velocity of the reaction was the same for three substrates, $MgIDP^-$, $MgUDP^-$ and $MgGDP^-$. However, the preliminary results of isotope exchange experiments reported in this chapter now makes the existence of a high enery enzyme-Pi complex seem unlikely.

A suggestion of what causes kinetic plots to become linear in the aged enzyme comes from a comparison of the values in Table IV.3, which are the combination constants for the reaction of substrate with the native enzyme without $MgATP^{2-}$, and the Michaelis constants for substrate reaction with the aged enzymes in the presence and absence of $MgATP^{2-}$. From these values it may be seen that for the aged enzyme plus $MgATP^{2-}$, the value of K_a, the Michaelis constant for the combination of substrate, is comparable to the value of the combination constant, K_{al}, which describes the combination of the

TABLE IV.3. : Values of the Michaelis constants obtained with the aged enzyme in the presence and absence of $MgATP^{2-}$ with the combination constants obtained with the native enzyme in the absence of $MgATP^{2-}$.

Kinetic Constant[a]	Weighted Mean Value (mM)	Number of Experiments
K_{a1}[b]	0.55 ± 0.19	4
K_{a2}	3.97 ± 0.33	4
K_a[c]	3.86 ± 1.17	3
K_a[d]	0.60 ± 0.02	9

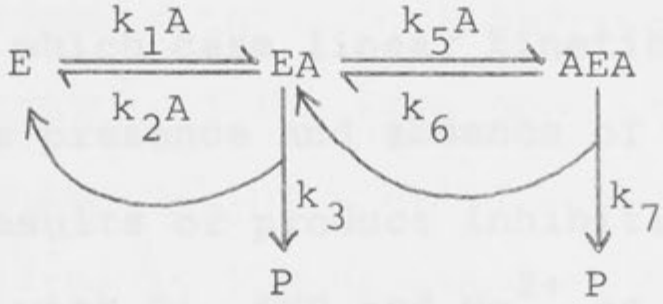
[a] K_{a1} and K_{a2} are combination constants and the K_a's are Michaelis constants for the combination of substrate with the enzyme.

[b] Values for K_{a1} and K_{a2} are for the native enzyme in the absence of $MgATP^{2-}$, and are taken from Table II.2 (Chapter II).

[c,d] Michaelis constants for the combination of substrate with the aged enzyme in the absence and presence of $MgATP^{2-}$, respectively.

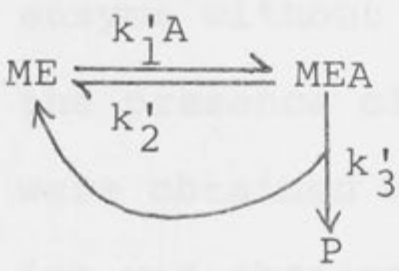
first molecule of substrate to the native enzyme in the absence of modifier. Further, the Michaelis constant for the combination of substrate with the aged enzyme in the absence of modifier (K_a) is comparable to the combination constant, K_{a2}, which describes the combination of the second molecule of substrate to the EA form of the native enzyme in the absence of modifier. These relationships are illustrated in schemes (IV), (V) and (VI).

Native enzyme, no activator



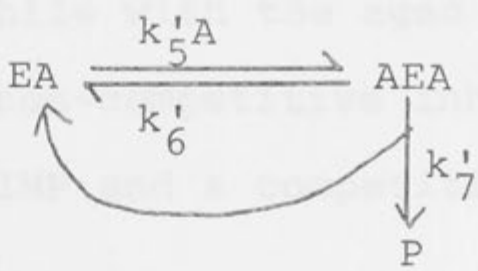
(IV)

Aged enzyme, activator present



(V)

Aged enzyme, no activator



(VI)

However, to adequately explain the results with the aged enzyme with no activator, it would be necessary to assume that the first molecule of A bound very tightly

relative to the second, and that the rate of breakdown of the EA complex to give products was negligible relative to k_7'. Thus the first molecule of A would be acting as an activator rather than a substrate for the reaction. The above possibilities have yet to be further investigated.

Summary

Nucleoside diphosphatase exhibits linear kinetics when the native enzyme is studied in the presence of high concentrations of modifier ($MgATP^{2-}$), and after ageing, in which case linear kinetics are obtained both in the presence and absence of modifiers.

The results of product inhibition studies have shown that with Pi, IMP and Mg^{2+} as product inhibitors, all non-competitive inhibition patterns were obtained for both the native enzyme plus modifier and the aged enzyme without modifier, while with the aged enzyme in the presence of modifier, non-competitive inhibitions were obtained with Pi and IMP and a competitive inhibition was obtained with Mg^{2+}.

Isotope exchange of Pi^{32} into IDP in the absence of IMP occurred for the aged enzyme both in the absence and presence of modifier, but could not be demonstrated for

the native enzyme plus modifier. Further, no exchange of IMP-8-C^{14} into IDP could be demonstrated.

In order to explain the experimental results, an ordered sequence of product release has been postulated with Pi release first, followed by IMP, and Mg^{2+} being the last product to be released. For this order of release, it has been necessary to postulate a dead-end E-IMP complex for the results obtained with the aged enzyme in the presence of modifier, while the results obtained with the native enzyme plus modifier and of the aged enzyme without modifier require in addition, an isomerization of the free form of enzyme.

Comparison of the kinetic constants obtained from the aged enzyme in the presence of $MgATP^{2-}$ have indicated that the Michaelis constant, K_a, is approximately three times larger than K_{ia}, the dissociation constant for the enzyme-MgIDP complex.

REFERENCES

Albers, R.W., Fahn, S., and Koval, G.J. (1963), Proc. Natl. Acad. Sci. U.S.A., 50, 474.

Andrews, P. (1964), Biochem. J., 91, 222.

Ankel, H., Ankel, E., and Feingold, D.S. (1966), Biochemistry, 5, 1864.

Atkinson, D.E. (1966), Ann. Rev. Biochem., 35, 85.

Atkinson, D.E., Hathaway, J.A., and Smith, E.C. (1965), J. Biol. Chem., 240, 2682.

Beck, W.S. (1967), J. Biol. Chem., 242, 3148.

Berger, L. (1956), Biochim. Biophys. Acta, 20, 23.

Bock, R.M., Ling, N.-S., Morell, S.A., and Lipton, S.H. (1956), Arch. Biochem. Biophys., 62, 253.

Botts, D.J. (1958), Trans. Faraday Soc., 54, 593.

Bresnick, E., Thompson, U.B., and Lyman, K. (1966), Arch. Biochem. Biophys., 114, 352.

Bridger, W.A., and Cohen, L.H. (1968), J. Biol. Chem., 243, 644.

Brown, W.E.L., and Hill, A.V. (1922), Proc. Roy. Soc. London, B94, 297.

Buzzee, D.H., and Levin, A.P. (1968), Biochem. Biophys. Res. Commun., 30, 673.

Byrne, W.L. (1961) In : The Enzymes, Boyer, P.D., Lardy, H.A., and Myrback, K., eds., Vol. 5, 2nd ed., New York, Academic Press, p.73.

Changeux, J.-P. (1961), Cold Spr. Harb. Symp. Quant. Biol., 26, 313.

Changeux, J.-P. (1963), Cold Spr. Harb. Symp. Quant. Biol., 28, 497.

Changeux, J.-P., Gerhart, J.C., and Schachman, H.K. (1968), Biochemistry, 7, 531.

Changeux, J.-P., and Rubin, M.M. (1968), Biochemistry, 7, 553.

Cho-Chung, Y.S., and Pitot, H.C. (1967), J. Biol. Chem., 242, 1192.

Cleland, W.W. (1963a), Biochim. Biophys. Acta, 67, 104.

Cleland, W.W. (1963b), Biochim. Biophys. Acta, 67, 173.

Cleland, W.W. (1963c), Biochim. Biophys. Acta, 67, 188.

Cleland, W.W. (1963d), Nature, 198, 463.

Cleland, W.W. (1967), Adv. in Enzymol., 29, 1.

Cohn, M. (1963), Biochemistry, 2, 623.

Cotton, R.G.H., and Gibson, F. (1965), Biochim. Biophys. Acta, 100, 76.

Cotton, R.G.H., and Gibson, F. (1967), Biochim. Biophys. Acta, 147, 222.

Dalziel, K. (1958), Trans. Faraday Soc., 54, 1247.

Datta, P., Gest, H., and Segal, H.L. (1964), Proc. Natl. Acad. Sci., Wash., 51, 125.

Doy, C.H. (1968), Proc. Australian Biochem. Soc., 12th annual meeting, p.69.

Farago, A., and Denes, G. (1967), Biochim. Biophys. Acta, 136, 6.

Frieden, C. (1964), J. Biol. Chem., 239, 3522.

Frieden, C. (1967), J. Biol. Chem., 242, 4045.

Frieden, C., and Colman, R.F. (1967), J. Biol. Chem., 242, 1705.

Gerhart, J.C., and Schachman, H.K. (1968), Biochemistry, 7, 538.

Gibson, D.M., Ayengar, P., and Sanadi, D.R. (1955), Biochim. Biophys. Acta, 16, 536.

Gregolin, C., Ryder, E., Warner, R.C., Kleinschmidt, A.K., Chang, H.-C., and Lane, M.D. (1968), J. Biol. Chem., 243, 4236.

Gregory, J.D. (1955), Fed. Proc., 14, 221.

Haber, J.E., and Koshland, D.E. (1967), Proc. Natl. Acad. Sci. U.S.A., 58, 2087.

Hass, L.F., and Byrne, W.L. (1960), J. Am. Chem. Soc., 82, 947.

Hayaishi, O. (1965), personal communication.

Helmreich, E., and Cori, C.F. (1964), Proc. Natl. Acad. Sci. U.S.A., 52, 647.

Helmreich, E., and Cori, C.F. (1964), Proc. Natl. Acad. Sci., Wash., 51, 131.

Heppel, L.A., Strominger, J.L., and Maxwell, E.S. (1959), Biochim. Biophys. Acta, 32, 422.

Hori, M., Gadd, R.E.A., and Henderson, J.F. (1967), Biochem. Biophys. Res. Commun., 28, 616.

Horn, A., Bornig, H., and Thiele, G. (1967), European J. Biochem., 2, 243.

Hsu, R.Y., Cleland, W.W., and Anderson, L. (1966), Biochemistry, 5, 799.

Hurlbert, R.B. (1957) In : Methods in Enzymology, Colowick, S.P., and Kaplan, N.O., eds., Vol. 3, New York, Academic Press, p.785.

Josan, V., and Krishnan, P.S. (1968), Biochem. Biophys. Res. Commun., 31, 299.

Kachmar, J.F., and Boyer, P.D. (1953), J. Biol. Chem., 200, 669.

Kerson, L.A., and Appel, S.H. (1968), J. Biol. Chem., 243, 4279.

Kirschner, K., Eigen, M., Bittman, R., and Voigt, B. (1966), Proc. Natl. Acad. Sci. U.S.A., 56, 1661.

Kirtley, M.E., and Koshland, D.E. (1967), J. Biol. Chem., 242, 4192.

Kornfeld, R. (1967), J. Biol. Chem., 242, 3135.

Kornfield, S. (1965), Federation Proc., 24, 536.

Kornfield, S., Kornfield, R., Neufeld, E.F., and O'Brien, P.J. (1964), Proc. Natl. Acad. Sci. U.S.A., 52, 371.

Koshland, D.E., Nemethy, G., and Filmer, D. (1966), Biochemistry, 5, 365.

Kowalik, J., and Morrison, J.F. (1968), Mathl. Biosci., 2,

Krebs, H.A., Newsholme, E.A., Speake, R., Gascoyne, T., and Lund, P. (1964), Advan. Enzyme Regulation, 2, 71.

Kunitz, M. (1952), J. Gen. Physiol., 35, 423.

Kutzbach, C., and Stokstad, E.L.R. (1967), Biochim. Biophys. Acta, 139, 217.

Kvamme, E., Tveit, B., and Svenneby, G. (1965), Biochem. Biophys. Res. Commun., 20, 566.

Larsson, A., and Reichard, P. (1966), J. Biol. Chem., 241, 2533.

Lindell, T.J., and Stellwagen, E. (1968), J. Biol. Chem., 243, 907.

Long, C.W., and Pardee, A.B. (1967), J. Biol. Chem., 242, 4715.

Lowry, O.H., and Passoneau, J.V. (1966), J. Biol. Chem., 241, 2268.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951), J. Biol. Chem., 193, 265.

MacElroy, R.D., Johnson, E.J., and Johnson, M.K. (1968), Biochem. Biophys. Res. Commun., 30, 678.

Maeba, P., and Sanwal, B.D. (1968), J. Biol. Chem., 243, 448.

Maehly, A.C. (1955) In : Methods in Enzymology, Colowick, S.P., and Kaplan, N.O., eds., Vol. 2, New York, Academic Press, p.357.

Magasanik, B., Moyed, H.S., and Gehring, L.B. (1957), J. Biol. Chem., 226, 339.

Maley, F., and Maley, G.F. (1965), J. Biol. Chem., 240, PC3226.

Martin, R.G. (1963), J. Biol. Chem., 238, 257.

Mayer, R.M., and Ginsburg, V. (1965), J. Biol. Chem., 240, 1900.

McClintock, D.K., and Markus, G. (1968), J. Biol. Chem., 243, 2855.

Melo, A., and Glaser, L. (1965), J. Biol. Chem., 240, 398.

Michaelis, L., and Menten, M.L. (1913), Biochem. Z., 49, 333.

Monod, J., Changeux, J.-P., and Jacob, F. (1963), J. Mol. Biol., 6, 306.

Monod, J., and Jacob, F. (1961), Cold Spr. Harb. Symp. Quant. Biol., 26, 389.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), J. Mol. Biol., 12, 88.

Morgan, H.E., and Parmeggiani, A. (1964), J. Biol. Chem., 239, 2440.

Morrison, J.F. (1965), Aust. J. Sci., 27, 317.

Morrison, J.F. (1968), Anal. Biochem., 24, 106.

Morrison, J.F., and James, E. (1965), Biochem. J., 97, 37.

Morrison, J.F., O'Sullivan, W.J., and Ogston, A.G. (1961), Biochim. Biophys. Acta, 52, 82.

Morrison, J.F., and Uhr, M.L. (1966), Biochim. Biophys. Acta, 122, 57.

Murphy, T.A., and Wyatt, G.R. (1965), J. Biol. Chem., 240, 1500.

Nierlich, D.P., and Magasanik, B. (1965), J. Biol. Chem., 240, 358.

Nichol, L.W. (1965), Aust. J. Sci., 27, 242.

Nichol, L.W., Jackson, W.J.H., and Winzor, D.J. (1967), Biochemistry, 6, 2449.

Noda, L., Kuby, S.A., and Lardy, H.A. (1954), J. Biol. Chem., 209, 203.

Noltmann, E.A., Gubler, C.J., and Kuby, S.A. (1961), J. Biol. Chem., 236, 1225.

Novikoff, A.B., and Heus, M. (1963), J. Biol. Chem., 238, 710.

Okazaki, R., and Kornberg, A. (1964), J. Biol. Chem., 239, 275.

O'Sullivan, W.J., and Cohn, M. (1966), J. Biol. Chem., 241, 3104.

O'Sullivan, W.J., and Perrin, D.D. (1964), Biochemistry, 3, 18.

Pierard, A. (1966), Science, 154, 1572.

Plaut, G.W.E. (1963) In : Methods in Enzymology, Colowick, S.P., and Kaplan, N.O., eds., Vol. 6, New York, Academic Press, p.231.

Preiss, J., Shen, L., and Partridge, M. (1965), Biochem. Biophys. Res. Commun., 18, 180.

Rosen, O.M., Copeland, P.L., and Rosen, S.M. (1967), J. Biol. Chem., 242, 2760.

Rosenbloom, J., Sugimoto, E., and Pizer, L.I. (1968), J. Biol. Chem., 243, 2099.

Rothman, L.B., and Cabib, E. (1967), Biochemistry, 6, 2107.

Sanwal, B.D., and Cook, R.A. (1966), Biochemistry, 5, 886.

Sanwal, B.D., Stachow, C.S., and Cook, R.A. (1965), Biochemistry, 4, 410.

Scatchard, G. (1949), Ann. N.Y. Acad. Sci., 51, 660.

Schramm, V.L., and Morrison, J.F., Biochemistry, in the press.

Scrutton, M.C., and Utter, M.F. (1967), J. Biol. Chem., 242, 1723.

Setlow, B., and Lowenstein, J.M. (1967), J. Biol. Chem., 242, 607.

Smiley, K.L., and Suelter, C.H. (1967), J. Biol. Chem., 242, 1980.

Smith, R.M., and Alberty, R.A. (1956), J. Am. Chem. Soc., 78, 2376.

Soldin, S.J., and Balinsky, D. (1968), Biochemistry, 7, 1077.

Stadtman, E.R. (1966), Adv. Enzymol., 28, 41.

Stadtman, E.R., Cohen, G.N., Le Bras, G., and de Robichon-Szulmajster, H. (1961), J. Biol. Chem., 236, 2033.

Stein, A.M., Kirkman, S.K., and Stein, J.H. (1967), Biochemistry, 6, 3197.

Steinmetz, M.A., and Deal, W.C. (1966), Biochemistry, 5, 1399.

Strominger, J.L., Heppel, L.A., and Maxwell, E.S. (1954), Arch. Biochem., 52, 488.

Sugimoto, E., and Pizer, L.I. (1968a), J. Biol. Chem., 243, 2081.

Sugimoto, E., and Pizer, L.I. (1968b), J. Biol. Chem., 243, 2090.

Umbarger, H.E. (1961), Cold Spr. Harb. Symp. Quant. Biol., 26, 301.

Vagelos, P.R., Alberts, A.W., and Martin, D.B. (1963), J. Biol. Chem., 238, 533.

Walaas, E. (1958), Acta Chem. Scand., 12, 528.

Weber, G., and Anderson, S.R. (1965), Biochemistry, 4, 1942.

Weber, K. (1968a), J. Biol. Chem., 243, 543.

Weber, K. (1968b), Nature, 218, 1116.

Webster, R.E., Nelson, C.A., and Gross, S.R. (1965), Biochemistry, 4, 2319.

Wiley, D.C., and Lipscomb, W.N. (1968), Nature, 218, 1119.

Worcel, A., Goldman, D.S., and Cleland, W.W. (1965), J. Biol. Chem., 240, 3399.

Yamazaki, M., and Hayaishi, O. (1965), J. Biol. Chem., 240, PC2761.

Yamazaki, M., and Hayaishi, O. (1968), J. Biol. Chem., 243, 2934.

Yoshino, M., Ogasawara, N., Suzuki, N., and Kotake, Y. (1967), Biochim. Biophys. Acta, 146, 620.

Zarlengo, M.N., Robinson, G.W., and Burns, R.O. (1968), J. Biol. Chem., 243, 186.